A mosaic analysis in *Drosophila* fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF

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Expression of the gene encoding the antifungal peptide Drosomycin in *Drosophila* adults is controlled by the Toll signaling pathway. The Rel proteins Dorsal and DIF (Dorsal-related immunity factor) are possible candidates for the transactivating protein in the Toll pathway that directly regulates the *drosomycin* gene. We have examined the requirement of Dorsal and DIF for *drosomycin* expression in larval fat body cells, the predominant immune-responsive tissue, using the yeast site-specific *flp/FRT* recombination system to generate cell clones homozygous for a deficiency uncovering both the *dorsal* and the *dif* genes. Here we show that in the absence of both genes, the immune-inducibility of *drosomycin* is lost but can be rescued by overexpression of either *dorsal* or *dif* under the control of a heat-shock promoter. This result suggests a functional redundancy between both Rel proteins in the control of *drosomycin* gene expression in the larvae of *Drosophila*. Interestingly, the gene encoding the antibacterial peptide Diptericin remains fully inducible in the absence of the *dorsal* and *dif* genes. Finally, we have used fat body cell clones homozygous for various mutations to show that a linear activation cascade Spaetzle→Toll→Cactus→Dorsal/DIF leads to the induction of the *drosomycin* gene in larval fat body cells.

Keywords: *Drosophila*/innate immunity/mosaic analysis/Rel proteins/Toll pathway

Introduction

A hallmark of the potent antimicrobial defense of *Drosophila* is the rapid synthesis by the fat body, following septic injury, of a battery of small-sized cationic peptides with a broad spectra of activities. Recent studies indicate that in *Drosophila* at least seven distinct peptides and their isoforms participate in this humoral immune response. Five of these peptides, Cecropins (Kylsten *et al.*, 1990), Diptericin (Wicker *et al.*, 1990), Drosocin (Bulet *et al.*,

1993), Attacin (Asling *et al.*, 1995) and insect Defensin (Dimarcq *et al.*, 1994) are only active against bacteria, whereas Drosomycin is only active against fungi (Fehlbaum *et al.*, 1994). The seventh and most recently discovered molecule, Metchnikowin, is active against both bacteria and fungi (Levashina *et al.*, 1995).

The study of the regulatory mechanisms controlling the rapid synthesis of these antimicrobial peptides after septic injury is an important challenge in the field (reviewed in Hoffmann and Reichhart, 1997). Analysis of the antimicrobial response in different mutant strains demonstrates that several regulatory pathways control antimicrobial peptide gene expression. This was first shown by the characterization of *immune deficiency* (*imd*), a recessive mutation that impairs the inducibility of the genes encoding antibacterial peptides in both larvae and adults, while only marginally affecting the inducibility of the antifungal peptide gene *drosomycin* (Lemaître *et al.*, 1995a). The *imd* gene, which has not yet been cloned, therefore encodes a component required for the antibacterial response. An extensive mutagenesis indicates that several other genes are involved in the control of the antibacterial peptide gene *dptericin* (D.Ferrandon, personal communication; Wu and Anderson, 1998). Recently, Williams *et al.* (1997) have shown that mutations in the *18-wheeler* gene, which encodes a Toll-like receptor, specifically alter the inducibility in larvae of the two other antibacterial peptide genes *cecropin* and *attacin*. This study therefore points to a complex network regulating the antibacterial response. Finally, a genetic approach performed in adults has also shown that the well-characterized Toll pathway controls the expression of the antifungal peptide gene *drosomycin* and is also involved in the control of some of the antibacterial peptide genes (e.g. *cecropin* and *attacin*; Lemaître *et al.*, 1996).

During embryonic dorsoventral patterning in *Drosophila*, the Toll (TL) receptor is thought to be activated by a processed form of the Spaetzle (Spz) protein. The activation of TL subsequently leads, via the cytoplasmic proteins Tube (Tub) and Pelle (Pll), to the degradation of the inhibitor protein Cactus (Cact) and the release of the Relish (Rel) protein Dorsal (Dl), which translocates into the nucleus and functions as a transcription factor (reviewed in Belvin and Anderson, 1996). Interestingly, this signaling pathway shares striking structural and functional similarities with the activation cascade of the Rel protein NF- κ B in cytokine-induced immune responses in mammals (reviewed in Belvin and Anderson, 1996). These similarities also extend to the *Drosophila* antimicrobial response: in adult flies, septic injury leads to the activation of TL, presumably by the *spz* gene product, and subsequently, via a Pll- and Tub-mediated cascade, to the degradation of Cact and the induction of the *drosomycin* gene (Lemaître *et al.*, 1996; Nicolas *et al.*, 1998).

In view of the parallels between the control of dorsoventral patterning and the induction of an antimicrobial response in *Drosophila*, it came as a surprise when early observations noted that in *dl*⁻ mutants, which lack a functional Rel protein Dl, the *drosomycin* gene (and those of all the other known antimicrobial peptides) remained fully inducible by an immune challenge (Lemaitre *et al.*, 1995b). This result indicated that either Dl was not the transactivator in this system, in contrast to the regulation of the dorsoventral target genes, or that the control of antimicrobial peptide genes was redundant and that other Rel proteins could substitute for Dl in *dl*⁻ mutants. An obvious candidate for such a redundant factor is the Rel protein DIF (for Dorsal-related immunity factor) characterized by Ip *et al.* (1993), which shares both sequence similarities and several significant biological features with Dl: the *dl* and the *dif* genes are both expressed in fat body cells, the major site of synthesis of antimicrobial peptides, and their expression is upregulated by immune challenge; the Dl and the DIF proteins are both translocated from the cytoplasm into the nucleus after septic injury; in transfection experiments with immune-responsive cell lines, both *dl* and *dif* expression vectors lead to the expression of antimicrobial peptide genes; and finally, both Dl and DIF bind to similar Rel binding sites that are present in the promoters of all genes encoding antimicrobial peptides (Ip *et al.*, 1993; Reichhart *et al.*, 1993; Petersen *et al.*, 1995; Gross *et al.*, 1996).

dif mutants have not been reported to date. However, the *dif* and *dl* genes, which map within 7 kb at position 36C on the left arm of the second chromosome, are both uncovered by a small deficiency (R. Steward, personal communication). To study the roles of *dl* and *dif* genes in the regulation of the antimicrobial peptide genes, we have developed a strategy for producing mitotic cell clones lacking both Dl and DIF activity using this deficiency and the yeast site-specific *flp/FRT* recombination system (Xu and Rubin, 1993). The induction of the antimicrobial peptide genes can, thus, be analysed in clones carrying the deficiency uncovering both the *dl* and *dif* genes. To determine the individual contributions of Dl and DIF to antimicrobial peptide gene expression, we used *dl* and *dif* transgenes under the control of a heat-shock promoter to reintroduce Dl or DIF activity into these clones.

Since our mosaic analysis was carried out with fat body cells of third instar larvae, and our previous studies had been performed on adult insects (Lemaitre *et al.*, 1996), we have also now carried out experiments on the induction of antimicrobial peptide genes in wild-type and dorsoventral mutant larvae. We now show that major similarities exist between the control of antimicrobial peptide gene expression in larvae and adults. In particular, the *drosomycin* gene is under the control of the dorsoventral pathway genes in larvae as well as in adults. Furthermore, as in the adults, the *imd* gene product is required in larvae for the induction of the antibacterial peptide genes. Our mosaic analysis in larval fat body shows that the control of *drosomycin*, but not that of *diptericin* in larvae, requires the Rel proteins Dl and DIF and that the two Rel proteins function redundantly *in vivo* to direct the expression of the *drosomycin* gene. Finally, using fat body cell clones homozygous for various mutants, we also show that a linear activation cascade Spz→Tl→Cact→Dl/DIF leads

to the induction of the *drosomycin* gene in larval fat body cells.

Results

Expression of antimicrobial peptide genes in larvae mutant for the Tl pathway

We have analysed the immune-inducibility of the genes encoding Drosomycin, Diptericin, Cecropin and Attacin in larvae carrying mutations in the Tl signaling pathway and in the *imd* gene. Two types of dorsoventral mutations were analysed: (i) loss-of-function mutations in *spz*, *Tl* and *dl* that block the Tl pathway in embryos and (ii) a gain-of-function mutation in *Tl* (*Tl*^D) and a loss-of-function mutation in *cact* that are strongly ventralizing. For this analysis, we extracted RNA from pools of unchallenged and 6 h-bacteria-challenged third instar larvae, and probed Northern blots with the corresponding radioactive cDNAs. The data were analysed by phosphoimaging and the results are plotted in Figure 1A.

A striking observation is that the *drosomycin* gene is strongly expressed in *Tl*^D gain-of-function and *cact*-deficient mutants in the absence of immune challenge. The level of *drosomycin* expression is significantly higher in *Tl*^D mutants than that induced by immune challenge in wild-type larvae. In addition, pricking the *Tl*^D gain-of-function mutants significantly increases the level of *drosomycin* expression over that of constitutive expression. This result indicates that in larvae, the activation of the Tl pathway in *Tl*^D mutant is sufficient to trigger a higher *drosomycin* expression than in adults (Lemaitre *et al.*, 1996). Figure 1A also shows that the induction of the *drosomycin* gene is lowered in *spz*-deficient larvae. The effect of this mutation, however, is less marked in larvae than previously seen in adults (Lemaitre *et al.*, 1996). The requirement of the Tl pathway for *drosomycin* expression is further illustrated by the observation that the inducibility of the *drosomycin* gene is also lowered in hypomorphic mutants of the *Tl* gene compared with wild-type larvae. Again, the effect of this reduction of the level of inducibility is less marked than in adults (Lemaitre *et al.*, 1996). However, this latter result could also be explained by the fact that the hypomorphic combination which we used, *Tl*⁶³²/*Tl*^{1-RXA}, is temperature sensitive and that the larvae are kept only at the restrictive temperature for 2 days, compared with 5 days for adults.

To ascertain the difference in Tl requirement between larvae and adults, we have corroborated our preceding data with a transgenic approach: we measured β-galactosidase activity 6 h after bacterial challenge in larvae and adults carrying a *drosomycin-lacZ* reporter gene combined with either the *Tl* or the *spz* mutations. These quantitative measurements (Figure 1B) clearly parallel our Northern blot analysis, indicating that (i) the induction of the *drosomycin* gene is lowered in *Tl*- and *spz*-deficient larvae and that (ii) the requirement of the Tl pathway for *drosomycin* induction is less marked in larvae than in adults (2-fold reduction in *spz*⁻ larvae versus 4-fold in *spz*⁻ adults).

As we had observed earlier for adults (Lemaitre *et al.*, 1996), the induction of the *drosomycin* gene is not affected in larvae deficient for the *dl* gene (Figure 1A), indicating that this Rel protein is either not involved in the transcrip-

Fig. 1. Analysis of antimicrobial gene expression in wild-type and dorsoventral mutant larvae. **(A)** The signals on several Northern blots were quantified using a bio-imager system. In each experiment, the signals of immune gene expression were normalized with the corresponding value of the rp49 signal. The levels of expression in 6 h-bacteria-challenged wild-type larvae were standardized as 100 and the results are given as relative activity (percent). Each bar corresponds to an independent experiment comprising 20 individuals. Analyses of Northern blots for *drosomycin*, *diptericin*, *cecropin* and *attacin* gene expression are presented. **(B)** Induction of the *drosomycin-lacZ* reporter gene in 6 h-bacteria-challenged wild-type, *Tl^R* and *spz^R* larvae and adults. Results are the mean of four measurements with the standard deviation in parentheses. C, unchallenged larvae; 6 h, 6 h after bacterial challenge. *Or^R*, Oregon R; *dl^R*, *dl¹/dl¹*; *Tl^R*, *Tl⁶³²/Tl¹RXA*; *spz^R*, *spz^{rm7}/spz^{rm7}*; *imd*, *imd/imd*; *imd; spz^R*, *imd; spz^{rm7}/spz^{rm7}*; *Tl^D*, *Tl^{10B}/+*; *cact*, *cact^{A2}/cact^{D13}*.

tional control of *drosomycin* or that another Rel protein(s) can substitute for its function. Finally, the *drosomycin* gene retains its inducibility in *imd* mutant larvae. However, when taking into account all the data which we obtained from various experimental series, we now conclude that the level of induction of the *drosomycin* gene is slightly reduced (approximately one third) in *imd* larvae (this study) and in *imd* adults (unpublished data). This weak effect of the *imd* mutation, which we had not formally noticed in our previous studies, suggests that the *imd* gene product also slightly participates in the regulation of the *drosomycin* gene.

In contrast to the *drosomycin* gene, the genes encoding the antibacterial peptides Diptericin, Cecropin and Attacin are not constitutively expressed in *Tl^D* gain-of-function mutant larvae, as seen in Figure 1A. The *diptericin* gene is also fully inducible in larvae deficient for the *spz* and *Tl* genes. These data indicate that *diptericin* induction in larvae is not dependent on the *Tl* pathway. *Diptericin* induction, however, is clearly dependent on the *imd* gene, since in *imd* mutants the level of *diptericin* induction by septic injury is dramatically reduced. The expression patterns observed for *cecropin* and *attacin* were somewhat different from those of *diptericin*, as the full induction of

Fig. 2. Clones of *Act5C>nuc-lacZ* expressing cells following *flp*-mediated recombination. *nuc-lacZ* expression in a wing imaginal disc (A) and fat body (B) were obtained from a late third instar *hsFLP12; Act5C>Draf⁺>nuc-lacZ* larva which had been heat-shocked for 30 min at 37°C at embryonic stage 4–6 h after egg-laying. A single patch of several hundred contiguous cells expressing *lacZ* can be seen in the posterior compartment of the wing disc (A). In contrast, the fat body in (B) is peppered with small patches of *lacZ* expression which rarely exceed four cells. Patches of stained cells resulting from clonal derivatives of single cells were observed in the fat body when the heat shock was administered <8 h after egg-laying.

these two genes is affected in both *spz* and *imd* mutant larvae, indicating that they are regulated both by the Tl pathway and the *imd* gene product. A similar situation was reported in adults (Lemaître *et al.*, 1996).

When the *imd* and *spz* mutations were combined, the effect on the level of induction of each antimicrobial peptide gene was stronger than in single mutants (Figure 1A). The absence of both pathways impaired all antimicrobial peptide gene expression, suggesting that the *imd* and Tl pathways together are essential for full antimicrobial resistance in *Drosophila* larvae. However, some expression of these genes remains detectable, suggesting that an additional pathway(s) may participate in the control of antimicrobial peptide gene expressions.

Mosaic analysis in larval fat body of Tl and *cact* gene function in antimicrobial peptide gene expression

Strong loss-of-function mutations in several genes of the Tl pathway decrease larval viability; this is particularly the case for null mutations in the *Tl* and *cact* genes (Gertulla *et al.*, 1988; Roth *et al.*, 1991). To analyse the regulation of the immune-induced expression of antimicrobial peptides in the fat body carrying null alleles of these genes, we generated mosaic animals in which somatic cell clones lack functional copies of either the *Tl* or the *cact* genes using the yeast site-specific recombination *flp/FRT* system.

To our knowledge, this method had not previously been applied to the larval fat body, and we first determined the parameters for clonal analysis in this tissue. For this, we used the FLP-out technique to heritably activate, in any cell, the coding sequence of nuclear localized β -galactosidase (Struhl and Basler, 1993). This method generates cell clones which constitutively express a nuclearly localized β -galactosidase (*nuc-lacZ*) under the control of a constitutive *actin5C* promoter only after a heat shock has induced FLP-mediated recombination between *cis*-acting FRTs. We subjected embryos aged 0–12 h (eggs were collected

Fig. 3. General genetic scheme of crosses for producing somatic clones in larval fat body using the yeast site-specific *flp/FRT* recombination system. Larval mosaic clones were generated using the yeast site-specific *flp/FRT* recombination system (Xu and Rubin, 1993). *hs-flp* females (here *hs-flp* refers to either *hsFLP12* or *hsFLP1*) carrying appropriate balancers with larval marker were crossed to *FRT mutation/Balancer* males. Males carrying both the *hs-flp* transgene and *FRT mutation* were then mated in mass to *dip1-lacZ, drom-GFP; FRT PZ* females [*PZ* refers to either *l(2)06270* or *l(3)j5C2* and is used as cell marker]. The induction of mitotic recombination and the analysis of reporter gene expression occurred in three steps as follows: (1), 0–6 h embryos were subjected to a heat shock at 37°C for 1 h to induce *flp/FRT*-mediated mitotic recombination; (2), resulting female larvae of the appropriate phenotype (*hs-flp/dip1-lacZ, drom-GFP; FRT mutation/FRT PZ*) were selected; (3), these larvae were either directly observed (unchallenged larvae) or bacteria-challenged. Larvae were collected 16 h after bacterial challenge and analysed for reporter gene expression (in some experiments, when indicated only *drom-GFP* is used as reporter gene). Male larvae of the same genotype as female except for the *hs-flp* transgene were used as internal controls.

every 2 h) to a single heat shock (37°C for 30 min). The surviving individuals were dissected at the third larval instar and analysed for nuclear β -galactosidase activity in the fat body. Under these conditions, we observed nuclear staining in isolated cells regardless of the time of the heat shock. However, clusters of stained cells representing clonal derivatives of a single cell were only observed when the heat shock was administered <8 h after egg-laying. The number of patches generated was directly dependent on the intensity and the duration of the heat shock. Figure 2 shows the *nuc-lacZ* staining pattern observed in fat body and wing imaginal disc from a larva obtained after embryonic heat shock at 4–6 h after egg-laying. In contrast to imaginal discs (Figure 2A), the number of cells of the clusters expressing *nuc-lacZ* is small in the fat body and does not exceed four cells (Figure 2B). We also observed that, in contrast to imaginal disc cells, fat body cells can disperse throughout this tissue during morphogenesis.

Using the *flp/FRT* methodology (see Figure 3 for the description of the general genetic scheme), we next generated homozygous larval fat body cell clones carrying null alleles for the *cact* or *Tl* genes in a heterozygous background. A number of experiments were performed on fly lines carrying both *dip1-lacZ* and *drosomycin-green fluorescent protein* (*GFP*) reporter genes on the

X chromosome (referred to as *dipt-lacZ* and *drom-GFP*, respectively). The use of these two reporter genes which reproducibly mimic the expression of the endogenous genes (Reichhart *et al.*, 1992; Ferrandon *et al.*, 1998) allows a direct comparison of the expression of both the *dipterizin* and the *drosomycin* genes in the same fat body cell.

Figure 4A illustrates the expression of the *drom-GFP* reporter gene in mosaic unchallenged larvae homozygous for the *cact* null allele, *cact*^{D13}. Groups of cells with marked GFP fluorescence are apparent in the fat body of the live animal through the integument. In contrast, as control we never observed a fluorescent signal in males of the same genotype but lacking the *hs-flp* chromosome, or in females heterozygous for an *FRT* chromosome. Figure 4B further illustrates the dissected fat body of a mosaic larva with clusters of strongly positive cells. In these experiments, the cells expressing *drom-GFP* were mostly rounded (Figure 4C or D) and exhibited a tendency to dissociate from the fat body (data not shown), which is typical for fat body cells of *Tl*^D larvae (Lemaître *et al.*, 1995b). In the course of these studies, we never observed constitutive expression of GFP in any tissue other than the fat body. We have ascertained that the cells which strongly express the *drosomycin* reporter gene in unchallenged larvae correspond to homozygous *cact*^{D13} clones by using an additional cell marker. For this, we constructed a fly strain carrying an *FRT* chromosome containing a *PZ* enhancer trap insertion, *l(2)06270*, which directs *lacZ* gene expression in fat body cells. Mosaic clones were induced in larvae with the genotype *hs-flp/dipt-lacZ, drom-GFP; FRT cact*^{D13}/*FRT l(2)06270*. In these larvae, all cells express *lacZ* due to the *PZ* insertion, except for those homozygous for *cact*, which lack the *PZ* insertion as a result of the *flp/FRT*-mediated recombination. Figure 4C and D show a typical result with two groups of two cells that strongly express the *drom-GFP* reporter and do not stain for β -galactosidase either from the *PZ* insertion or from the *dipt-lacZ* reporter gene. This demonstrates that these cells indeed correspond to *cact*^{D13} homozygous cells derived from *FRT*-induced mitotic recombination and that the absence of *cact* leads to a constitutive expression of the *drosomycin* gene in larval fat body cells. Additionally, the mosaic cells deficient for *cact* do not express the *dipterizin* reporter gene in the absence of immune challenge (red dotted lines in Figure 4D).

We further analysed the expression of the *drosomycin* and the *dipterizin* genes in fat bodies of larvae containing cells homozygous for *Tl* null allele (*Tl*^{QRE}). Similarly with the use of the *FRT l(3)j5C2* fat body cell marker, we observed that the cells which failed to express the *drosomycin* reporter gene in challenged larvae (Figure 4E) correspond to homozygous *Tl* clones. *Tl* homozygous cells characterized by loss of *lacZ* expression of the *PZ* insertion (Figure 4F), clearly display lower levels of the *drom-GFP* reporter gene (Figure 4E). This demonstrates unambiguously the requirement of *Tl* for the *drosomycin* gene inducibility. We analysed the expression of the *drosomycin* and the *dipterizin* genes in the same fat body sample of a larvae containing cells homozygous for a *Tl* null allele. In contrast to *drom-GFP* (Figure 4G), we observed that the *dipt-lacZ* reporter gene remained

Fig. 4. Analysis of *drosomycin* and *dipterizin* reporter gene expression in larvae mosaic for *cact* and *Tl* null mutations. (A–D) Mosaics for a *cact* null allele, *cact*^{D13}. Mitotic recombination was induced in *hsFLP12/dipt-lacZ, drom-GFP; FRT cact*^{D13}/*FRT l(2)06270* larvae. (A) In the absence of immune challenge, strong fluorescence signals indicating constitutive *drom-GFP* expression are apparent only in the fat body through the integument. (B) A fat body fragment dissected from the larva shown in (A). Fluorescence is seen in cell patches in a pattern similar to that observed in Figure 2B. *Drom-GFP* (C) and *lacZ* (D) expression were examined in the same fat body fragment. Homozygous *cact*^{D13} cells do not carry the *PZ* element and do not stain for *lacZ* expression (D, red dotted lines). These cells constitutively express the *drom-GFP* reporter gene (C, red dotted lines) but do not express the *dipt-lacZ* reporter gene (D, red dotted lines; *lacZ* staining from *dipt-lacZ* is expected to be cytoplasmic). The twin-spot cells which carry two copies of the *PZ* exhibit a higher level of *lacZ* staining (D, black dotted lines). (E and F) Mosaics for a *Tl* null mutation, *Tl*^{QRE}. Mitotic recombination was induced in *hsFLP12/drom-GFP; FRT Tl*^{QRE}/*FRT l(3)j5C2* larvae. Reporter gene expression was analysed 16 h after immune challenge. Cells that express a lower level of the *drosomycin* reporter gene in challenged larvae (E, red dotted lines) correspond to homozygous *Tl* clones ascertained by the lack of *lacZ* expression (F, red dotted lines). Black dotted lines in (F) show wild-type twin spot cells. (G and H) Mitotic recombination was induced in *hsFLP12/dipt-lacZ, drom-GFP; FRT Tl*^{QRE}/*FRT l(3)j5C2* larvae. The mosaic cells failed to express the *drom-GFP* reporter gene (G, red dotted lines) but still expressed the *dipt-lacZ* gene after immune challenge (H, red dotted lines).

inducible in these *Tl* null homozygous cells as in wild-type cells (Figure 4H).

Altogether, this mosaic analysis confirms our results obtained by Northern blot analysis which showed that the *Tl* signaling pathway controls the expression of the *drosomycin* gene in larvae. Furthermore, these experiments show for the first time that the *cact* and *Tl* genes act in a cell-autonomous manner in the fat body, demonstrating that the *Tl* signaling pathway directly controls the expression of the *drosomycin* gene in this tissue.

The Rel proteins DIF and/or DI control the expression of the *drosomycin* gene but not that of *dipterizin*

We have extended the mosaic approach to the function of the Rel proteins DIF and DI in the control of antimicrobial gene expression in larvae. *Dif* and *dl* are located within 7 kb of each other at position 36C on the left arm of the second chromosome. *Df(2L)TW119* is a small deficiency (hereafter referred to as *TW119*) that was shown to uncover both the *dif* and the *dl* genes, together with several other lethal genes (Steward and Nusslein-Volhard, 1986). We have confirmed by PCR studies that neither *dif* nor *dl* sequences are present in this deficiency (data not shown). The *TW119* deficiency is embryonic lethal and we first determined if it would be possible to generate viable *TW119* homozygous cells (which, consequently, are devoid of *dif* and *dl*) in the fat body. We used the cell marker approach as described above with *cact* and compared the expression of the *lacZ* gene of the *PZ* insertion in female larvae of genotype *hsFLP12/+; FRT TW119/FRT l(2)06270* with that in male larvae of genotype *+/+; FRT TW119/FRT l(2)06270*, which differ by the absence of a heat-shock inducible *flp* recombinase gene. Whereas all fat body cells of males expressed the *lacZ* gene, fat body from females contained cell clones devoid of *lacZ* activity (data not shown, see also Figure 5A). These clones were homozygous for the *TW119* deletion. We did note, however, that the *TW119* cells were often smaller than the cells expressing β -galactosidase and that their number was lower than expected, suggesting that this deletion somehow affects cell growth or cell viability.

Figure 5B illustrates induction by bacterial challenge of the *drom-GFP* reporter gene in the fat body of a mosaic larva containing cells homozygous for the *TW119*. Importantly, we observed that homozygous *TW119* cells could not be induced to express (or at very low levels) the *drosomycin* reporter gene after septic injury, indicating that one or several genes present in the deleted sequence is (are) required for the induction of *drosomycin* (Figure 5B). In this experiment, we have ascertained that the cells that failed to express the *drosomycin* reporter gene in challenged larvae correspond to homozygous *TW119* clones by using the previously described *FRT l(2)06270* cell marker. In these larvae, all cells express *lacZ*, except for those homozygous for *TW119*, which lack the *PZ* insertion as a result of the *flp/FRT*-mediated recombination (Figure 5A). This mosaic expression of the *drom-GFP* reporter gene after immune challenge in *FRT TW119* mosaic was highly reproducible allowing for the unambiguous identification of *TW119* homozygous cells.

To facilitate the selection of mosaic larvae in subsequent experiments, we replaced the *FRT PZ* chromosome by a

Fig. 5. Analysis of *drosomycin* and *dipterizin* reporter gene expression in fat body mosaic for the *TW119* deficiency. (A and B) Mosaics for *TW119* deficiency and *l(2)06270* insertion that was used as cell marker. Mitotic recombination was induced in *hsFLP12/drom-GFP; FRT TW119/FRT l(2)06270* larvae. (A) The absence of *lacZ* staining (A, red dotted lines) reveals the presence of *TW119* homozygous cells. (B) *TW119* homozygous cells do not express the *drom-GFP* reporter gene after bacterial challenge (B, red dotted lines). (C and D) *TW119* clone and *cact*^{D13} twin-spot clone. Mitotic recombination was induced as described in *hsFLP12/dipt-lacZ, drom-GFP; FRT TW119/FRT cact*^{D13} larvae. Live *TW119* mosaic larvae were bacteria-challenged and reporter gene expression was analysed 16 h after immune challenge. The same fat body fragment was observed using epifluorescence microscopy (C) and then stained for *lacZ* activity (D). The expression of the *drom-GFP* gene was not induced in homozygous *TW119* cells (C, red dotted lines) whereas the *dipterizin* reporter gene remains inducible (D, red dotted lines). Note that a *cact* twin-spot clone displayed an higher level of *drom-GFP* reporter gene expression after immune challenge compared with the rest of the tissue (C, black dotted lines) and also expressed the *dipt-lacZ* reporter gene (D, black dotted lines). (E and F) *drom-GFP* expression in *TW119* mosaic fat body derived from bacteria-challenged larvae carrying either *hs-dif* (E) or *hs-dl* transgene (F). Mitotic recombination was induced in *hsFLP12/dipt-lacZ, drom-GFP; FRT TW119/FRT cact*^{D13}; *hs-dif* or *hs-dl/+*. Live mosaic larvae were then heat-shocked and challenged with bacteria 3 h later. *Drom-GFP* reporter gene expression was analysed 16 h after bacterial challenge. In *TW119* clones obtained from heat-shocked larvae lacking the *hs-dif* and *hs-dl* transgene, no *drom-GFP* expression was observed in *TW119* cells (data not shown). In immune-challenged mosaic larvae carrying the *hs-dif* (E) or *hs-dl* (F) transgenes, nearly all cells expressed the *drosomycin* reporter gene after heat shock. Black dotted lines in (E) and (F) indicate the *cact* twin-spot clones.

FRT cact^{D13} chromosome. Clones were generated by heat shock and live mosaic larvae could easily be scored under the epifluorescence microscope by the presence of cells which strongly expressed the *drom-GFP* reporter gene, as a result of the absence of Cact protein in these cells (see Figure 4A and B). In addition, even after septic injury, these cells exhibited a stronger *drom-GFP* gene expression than the other wild-type cells (black dotted lines in Figure 5C, E and F). Importantly for the following experiment, we observed a high frequency of *TW119* homozygous cells (detected by the lack of *drom-GFP* expression after immune challenge) in proximity to the *cact*^{D13} mosaic cells (a typical picture is shown in Figure 5C).

Fig. 6. *Tl* and *cact*-mediated *drom*-GFP reporter gene expression in *TW119* mosaic cells. (A and B) expression of the *drom*-GFP gene in *TW119* homozygous fat body cells from *Tl^{10B}* unchallenged larvae. Mitotic recombination was induced in *hsFLP1/dipt-lacZ, drom-GFP; FRT TW119/FRT l(2)06270; Tl^{10B}/+* larvae. The same fat body fragment was observed under epifluorescence microscope (B) and then stained for *lacZ* activity (A). Homozygous *TW119* cells which do not carry the PZ element do not stain for *lacZ* gene expression (A, red dotted lines) and do not express the *drom*-GFP reporter gene (B, red dotted lines). (C–F): expression of the *drom*-GFP gene in *cact^{D13}*, *TW119* homozygous cells from unchallenged (C, D) or immune-challenged (E, F) larvae. Mitotic recombination was induced in *hsFLP12/drom-GFP; FRT cact^{D13}, TW119/FRT l(2)06270*. The same fat body fragment was observed using epifluorescence microscopy (D, F) and then stained for *lacZ* activity (C, E). No expression of the *drom*-GFP reporter gene was detected in *cact^{D13}*, *TW119* homozygous cells derived from unchallenged (D) or challenged larvae (F). (G and H) *drom*-GFP expression in both *cact^{D13}*, *TW119* homozygous fat body cells from unchallenged larvae carrying an *hs-dl* transgene. Mitotic recombination was induced in *hsFLP1/dipt-lacZ, drom-GFP; FRT cact^{D13}, TW119/FRT l(2)06270; hs-dl/+*. Five hours after the heat-shock-induced overexpression of *dl* gene, *cact^{D13}*, *TW119* homozygous fat body cells [which do not stain for *lacZ* gene expression, (G), red dotted lines] express the *drosomycin* reporter gene in the absence of challenge (H, red dotted lines). Note that some wild-type cells show a weak expression of the *drosomycin* gene.

Interestingly, we observed that cells homozygous for the *TW119* deletion which fail to express the *drom*-GFP reporter gene still express wild-type levels of the *dipt-lacZ* reporter after septic injury (compare Figure 5C with D), indicating that neither the DI nor the DIF proteins are strictly required for the induction of *dipteracin*. This also demonstrates that the lack of inducibility of the *drom*-GFP gene in the *TW119* clones is not due to lethality since they can be induced to express the *dipteracin* reporter gene in the same experiments (Figure 5C).

We next examined whether overexpression of *dl* or *dif* could restore the immune-inducibility of *drosomycin* in the *TW119* clones. For this, we repeated the same analysis as above, except that a transgene containing either *dl* or *dif* under the control of a heat-shock promoter was used (see Materials and methods and legend to Figure 5 for details). As control, bacterial challenge did not induce reporter gene expression in homozygous *TW119* clones in heat-shocked larvae that did not carry the *hs-dif* or *hs-dl* transgenes (data not shown). We subsequently observed that overexpression of *dif* or *dl* under a heat-shock promoter did not significantly induce the expression of the *drosomycin* gene in the absence of an immune challenge (data not shown and below). Importantly, in immune-challenged mosaic larvae carrying the *hs-dif* or *hs-dl* transgenes, nearly all of the fat body cells express *drom*-GFP, indicating that overexpression of these Rel proteins can rescue the lack of *drosomycin* inducibility in homozygous *TW119* cells. We have compared these mosaic larvae with mosaic clones from sister larvae that lack the *hs-dif* or *hs-dl* transgenes and display a high percentage of cells which do not express the *drom*-GFP reporter gene in proximity to *cact* twin spot. We estimate that >80% of the *TW119* homozygous clones expressed the *drosomycin* reporter gene following heat-shock induction of the *hs-dif* or *hs-dl* transgenes (Figure 5E and F, respectively). Under these conditions we did not observe a difference between the

lines overexpressing *dl* and those expressing *dif*. These results show that overexpression of *dif* or *dl* in cells lacking DIF and DI activity is sufficient to restore the ability to strongly express *drosomycin* after an immune challenge. Additionally, under the conditions of these experiments, *dif* and *dl* both elicited similar effects on *drosomycin* gene expression.

The *Tl*-dependent induction of the *drosomycin* gene is mediated through the Rel proteins DIF and/or DI

The data presented above demonstrate that *drosomycin* gene expression in the fat body is controlled by the *Tl* and *cact* genes. In addition, they show that the deletion of both *dif* and *dl* prevents the activation of the *drosomycin* gene by septic injury. We next determined whether the *Tl*-dependent activation of the *drosomycin* gene in the larval fat body is mediated via the Rel proteins DI and/or DIF. We therefore generated *TW119* homozygous clones in larvae carrying a *Tl^D* gain-of-function allele of *Tl* which signal-independently activates the *Tl* pathway. The results of this experiment were clear: the *TW119* cell clones (identified as above by the absence of *lacZ* expression of the PZ cell marker) did not display constitutive (*Tl^D*-driven) expression of the *drosomycin* reporter (Figure 6A and B). This indicates that the control of *drosomycin* gene expression via the *Tl* receptor requires the products of either the *dl* and/or the *dif* genes.

We next determined if the constitutive activation of the *drosomycin* gene observed in *cact* null mutants was also mediated by the activity of DIF and/or DI. For this, we used meiotic recombination to construct an FRT chromosome carrying both the *cact^{D13}* mutation and the *TW119* deficiency. Thus, mosaic animals exhibited homozygous *cact*, *TW119* double mutant cells in a heterozygous context. As illustrated in Figure 6C and D, the *cact*, *TW119* homozygous cells fail to express the *drosomycin*

reporter gene in the absence of a septic injury. Even after septic injury, expression of *drosomycin* cannot be induced (Figure 6E and F), in contrast to *dipterocin* (data not shown). This result establishes that the effect of the *cact* null mutation on both the constitutive and the induced expression of the *drosomycin* gene is actually mediated by *dl* and/or *dif*.

This study and several previous reports (Lemaitre *et al.*, 1995b, 1996) have established that the expression of the *drosomycin* gene is not altered in *dl*⁻ mutants. However, our mosaic analysis clearly indicates that overexpression of *dl* in the absence of *dif* is sufficient for inducing *drosomycin* gene expression after immune challenge. In order to ascertain the potential role of *dl* in the control of this antifungal gene, we also examined whether *dl* overexpression could restore the constitutive expression of the *drosomycin* reporter gene in *cact*, *TW119* clones. A transgene carrying the *dl* gene under the control of a heat-shock promoter was used to reintroduce the *DL* protein. Figure 6G and H show that in the absence of an immune challenge, the expression of *dl* by the *hs-dl* transgene is sufficient to restore the constitutive expression of the *drom-GFP* reporter gene in *cact*, *TW119* cells. In contrast, little *drom-GFP* expression was observed after heat-shock-driven expression of *dl* in the other fat body cells, which are heterozygous for *cact*, *TW119*. This result demonstrates that, in the absence of the Cact and DIF proteins, the presence of the *DL* protein is able to transactivate the *drosomycin* gene in larval fat body cells.

Discussion

Either *Dif* or *DL* control the expression of the *drosomycin* gene in the larval fat body

Numerous studies in recent years have suggested that Rel proteins are involved in the immune response of *Drosophila* as they are in mammals (for a recent review see Hoffmann and Reichhart, 1997). This idea first stemmed from the observation that the genes encoding inducible antimicrobial peptides in insects contain upstream sequences similar to κB motifs (binding sites for NF- κB) which are mandatory for their inducibility by immune challenge (Engström *et al.*, 1993; Kappler *et al.*, 1993). The fat body, which is the predominant immune-responsive tissue in *Drosophila*, expresses at least three Rel proteins: *DL* (Steward, 1987; Reichhart *et al.*, 1993) (plus a splice isoform, *DL-B*; Gross *et al.*, 1999), *DIF* (Ip *et al.*, 1993) and *Relish* (Dushay *et al.*, 1996). *In vitro* studies have shown that *DL* and *DIF* can transactivate some of the antimicrobial peptide genes in blood cell lines (Petersen *et al.*, 1995; Gross *et al.*, 1996); however, to date, the role of these two Rel proteins in the humoral immune response of *Drosophila in vivo* had not been established. The present study demonstrates that *DIF* and *DL* control the expression of the *drosomycin* gene in the fat body of larvae: we show that cells homozygous for the *TW119* deletion, which uncovers the *dif* and *dl* genes, fail to express the *drosomycin* gene in response to septic injury, and that the overexpression of either *dif* or *dl* through a heat-shock promoter restores the inducibility of this gene in the same cells.

Our study points to the existence of a functional redundancy between these two transcription factors in

their regulation of antimicrobial gene expression in the larval fat body. The *dif* and *dl* genes are in close proximity (Ip *et al.*, 1993), suggesting that they result from a relatively recent duplication event. The Rel domains of the two proteins present a high level of sequence identity (48%; Ip *et al.*, 1993). Only the *dl* gene, however, is expressed in the early embryo where it regulates dorsoventral patterning. Recently, Stein *et al.* (1998) have shown that maternal expression of *dif* can partially rescue embryos from the consequences of a *dl* mutation, suggesting that *DIF* can partially substitute for *DL* in this context. Furthermore, these experiments showed that *dl*-deficient embryos rescued by *dif* exhibited dorsoventral polarity, which indicates that in these conditions *DIF* remained sensitive to the dorsoventral signaling cascade transmitted through *Tl*. Nevertheless, *DL* and *DIF* are not totally interchangeable in this context, as *dl*⁻ embryos were only partially rescued by expression of the *dif* gene (Stein *et al.*, 1998). This observation is consistent with *in vitro* data in the immune system showing that *DIF* and *DL* have somewhat different transactivating capabilities and do not bind κB -motifs present in the upstream regions of the genes encoding Cecropin and Dipterocin with the same affinities (Petersen *et al.*, 1995; Gross *et al.*, 1996). *DIF* and *DL* are good examples of two molecules resulting from the duplication of an ancestral gene which have acquired different functional properties during evolution. It will certainly be of interest to investigate their roles in other species. The promoter of the *drosomycin* gene reveals a complex pattern of multiple κB -sites (L. Michaut, personal communication) and their functional analysis should reveal how they contribute to the *DIF*- and *DL*-induced transcription of this gene.

The *Tl* pathway controls the expression of the *drosomycin* gene in both larvae and adults

In a previous study, we had reported that the products of the *spz*, *Tl*, *tub*, *p11* and *cact* genes are involved in the control of *drosomycin* gene expression in adult flies (Lemaitre *et al.*, 1996). The present analysis extends this study by showing that the *Tl* pathway is also functionally active in the fat body of third instar larvae and controls *drosomycin* expression.

The larval polyploid fat body cells differentiate from embryonic mesodermal cells whereas the adult fat body cells are derived from larval histoblasts; presumably from ad epithelial cells associated with the imaginal discs (Hoshizaki *et al.*, 1995). It was of interest therefore to compare the regulation of antimicrobial genes during an immune response in these relatively different cell types. Our results, which are mostly based on Northern blot analysis, point to an overall similar mode of regulation in larval and adult fat body cells. In essence, the *Tl* pathway controls *drosomycin* gene expression whereas the genes encoding the antibacterial peptides require the product of the *imd* gene (dipterocin) or a combination of the *imd* and *Tl* pathways (cecropin and attacin). These results are in keeping in larvae with a correlation between the impairment of antifungal gene induction and reduced resistance to fungal infection and, conversely, between the impairment of antibacterial gene induction and reduced resistance to bacterial infection (P. Manfrulli unpublished data). Northern blot analysis, furthermore, indicates that the

inducibility of the *drosomycin* gene in Tl pathway mutants is less dramatically affected in larvae than in adults. This suggests that another regulatory cascade might partially substitute for the Tl pathway in controlling *drosomycin* in larval fat body. *Drosophila* contains several Tl-like receptors (Mitcham *et al.*, 1996), including 18-Wheeler, which is reportedly involved in the control of *attacin* and, to a lesser extent, *cecropin* induction in larvae (Williams *et al.*, 1997). However, *18-wheeler* mutations do not seem to affect *drosomycin* expression (E.Eldon, personal communication; P.Manfrulli, unpublished data). The possible contribution of these receptors to the humoral immune response, and namely to the regulation of the *drosomycin* gene, awaits further investigation.

Finally, we have noted that in larvae, as in adults, the inducibility of the *drosomycin* gene is slightly reduced in *imd* mutants. This result, in conjunction with studies on *metchnikowin* gene expression, leads us to propose that each antimicrobial peptide gene is regulated by the relative dosage of inputs from several signaling cascades that are each triggered by distinct stimuli (e.g. distinct microbial patterns; Lemaitre *et al.*, 1997). Current programs of mutagenesis (D.Ferrandon, personal communication; Wu and Anderson, 1998) will contribute to the identification of new components of these cascades and help understand the cross-talk between distinct pathways.

The Tl signaling cascade regulates numerous functions in various tissues at several developmental stages: dorso-ventral axis formation (reviewed in Belvin and Anderson, 1996), proliferation of blood cells (Qiu *et al.*, 1998), muscle cell attachment (Halfon *et al.*, 1995), axon guidance (Rose *et al.*, 1997) and larval size (Letsou *et al.*, 1991). Through our *flp/FRT* approach, we have shown here that the *Tl* and *cact* genes function cell-autonomously to regulate the antifungal response in the larval fat body. This result definitively proves that the *drosomycin* gene expression is directly regulated by the Tl pathway in the fat body cells.

The Tl pathway controls the expression of the *drosomycin* gene in larvae via the DIF and DI proteins

An interesting result of this study is the observation that the constitutive expression of the *drosomycin* gene in *Tl^D* gain-of-function mutants is abolished in fat body cells deficient for the *dif* and *dl* genes. This result clearly shows that the Tl function in the immune response is mediated by DI and DIF. Similarly, the absence of constitutive expression of the *drosomycin* gene in *cact*, *TW119* clones demonstrates that DIF and DI are actually the Rel proteins sequestered by Cact that control *drosomycin* gene expression upon release from the inhibitor. This result is in agreement with a variety of previous studies that have shown that DI and DIF bind to Cact *in vitro* (Kidd, 1992; Lehming *et al.*, 1995; Tatei and Levine, 1995; Govind *et al.*, 1996). Under *in vitro* conditions, DI and DIF can form heterodimers (Gross *et al.*, 1996). However, our rescue experiments clearly show that DIF can function in the absence of DI to regulate *drosomycin* gene expression and the same holds true for DI in the absence of DIF. Finally, our data do not rule out the possibility that a fraction of the DIF or DI proteins are associated with other inhibitor proteins. The observation, however, that

the overexpression of *dl* in *cact*, *TW119* cell clones (which lack both Cact and DIF) in the absence of a septic injury leads to the transcription of the *drosomycin* reporter gene, indicates that Cact is necessary to retain DI in these conditions. Recently, Wu and Anderson (1998) have shown by immunolocalization experiments that a septic injury can induce nuclear translocation of DIF in larvae deficient for the Tl receptor whereas under the same conditions, DI remains cytoplasmic. They interpret their results by suggesting that the signaling pathway that targets Cact for degradation must discriminate between Cact/DI and Cact/DIF complexes. An alternative explanation is that a fraction of DIF, but not DI, could be complexed to another inhibitor, for instance to the ankyrin repeats of Relish, in the form of DIF/Relish heterodimers evocative of relA/p105 heterodimers in mammals. According to this hypothesis, this fraction of DIF could be translocated to the nucleus in *Tl⁻* mutants upon immune challenge.

Diptericin remains inducible in the absence of DIF and DI

Our data demonstrate that the gene encoding the antibacterial peptide Diptericin remains inducible in fat body cells homozygous for the *TW119* deficiency. This indicates that in contrast to *drosomycin*, *diptericin* expression does not require the Rel proteins DIF or DI. These results are in agreement with our earlier observations, based on a genetic analysis in adults (Lemaitre *et al.*, 1996), that *diptericin* expression is controlled by mechanisms largely different from those that regulate *drosomycin* expression. The promoter of the *diptericin* gene contains two identical κ B-sites which are mandatory for the expression of this gene (Kappler *et al.*, 1993; Meister *et al.*, 1994). Earlier studies from this laboratory have shown that the overexpression of *dl* in cultured cell lines can transactivate a reporter gene placed under the control of eight *diptericin* κ B-sites (Reichhart *et al.*, 1993). This result suggested that DI can play a role in the regulation of *diptericin*, at least in the malignant blood neoplasm-2 blood cell line. Our current observations, however, strongly suggest that another Rel protein is involved in the control of *diptericin* expression: Relish is obviously an excellent candidate. Altogether, we propose that the genes encoding the various antimicrobial peptides are controlled by different combinations of Rel-transactivating proteins that, in turn, are activated via distinct signaling cascades elicited by specific microbial populations (Lemaitre *et al.*, 1997).

A clonal analysis of the humoral antimicrobial response

The present report is the first study making use of the *flp/FRT* approach in larval fat body cells of *Drosophila*. As noted above, the larval fat body is a uniform tissue consisting of polyploid cells derived from embryonic mesoderm. The use of a cell marker expressing the *lacZ* gene has allowed us to visualize the clones generated after recombination. This clonal analysis indicates that the precursor cells of the larval fat body undergo only a limited number of divisions, in contrast to imaginal disc cells. According to the expression of *serpent* during embryogenesis (Riechmann *et al.*, 1998), ~52 progenitor cells of the fat body which are present in parasegment 4–13, undergo two rounds of cell division. Furthermore, the

cells derived from a given clone show a variable spatial distribution, suggesting the existence of extensive cell movements within the fat body.

The use of appropriate transgenes has allowed us to compare the expression of the *drosomycin* and the *dip-tericin* genes in the same cells. This mosaic approach is not quantitative but gives a qualitative indication of the expression, within the same fat body, of a given gene in cells that are homozygous or heterozygous for a regulatory mutation. This approach is, therefore, particularly well suited for the study of early lethal genes. In addition, mutations that affect larval viability frequently result in varying delays in larval development. It should be kept in mind that the inducibility of the *dip-tericin* gene, for instance, increases markedly in the course of the third larval instar stage (Meister and Richards, 1996), which is a major drawback for accurate comparisons between different individuals.

The existence of reporter genes for all antimicrobial peptides of *Drosophila* (J.L. Imler, personal communication) will enable the extension of the present studies to all these genes. A major application of the *flp/FRT* method is the possibility of performing F₁ genetic screens to identify lethal mutations leading to constitutive antimicrobial peptide gene expression. Such screens have already identified important genes that escaped detection in traditional screens (Xu and Rubin, 1993). This *flp/FRT* approach to the fat body is a welcome addition to the battery of genetic tools that make *Drosophila* a particularly attractive model for the molecular analysis of primordial innate immunity.

Materials and methods

Drosophila stocks

Fly cultures and crosses were grown on standard fly medium at 25°C, unless otherwise indicated. The wild-type stock was Oregon R. We constructed a transgenic strain (*drom-lacZ*) carrying a *drosomycin* reporter gene on a *w* X chromosome. The fusion gene contains 1.88 kb *XhoI*–*NheI* fragment of *drosomycin* upstream sequences fused to the bacterial *lacZ* coding region and was inserted into the pCasper transformation vector. The inducible expression of the *drom-lacZ* transgene is roughly identical to that of the resident *drosomycin* gene at the adult stage (data not shown). *dl*¹, *cact*^{A2}, *cact*^{D13}, *TI*⁶³², *TI*^{1-RXA}, *TI*^{9ORE}, *TI*^{10B}, *spz*^{m7} and *imd* mutant lines have been described elsewhere (Anderson and Nusslein-Volhard, 1984; Anderson et al., 1985; Lemaitre et al., 1995a,b). Mutants in *TI* have been reported to exhibit significant lethality during the larval stage (Gertulla et al., 1988). To obtain *TI* larvae and adults, we used two thermosensitive alleles of *TI* (*TI*⁶³² and *TI*⁴⁴⁴) which exhibit a strong phenotype only when raised at 29°C (Gertulla et al., 1988). *TI*-deficient mutants were reared at 18°C and shifted to 29°C at the second instar. *TI*^{9ORE} is a null embryonic lethal allele of *TI*. *cact*^{D13} is a null lethal allele of *cact* that contains a premature stop codon at amino acid 188 of the *cact* gene (Bergmann et al., 1996). *Df(2R)TW119*, (*TI*¹¹⁹) is a small embryonic lethal deficiency which uncovers the *dl* locus and at least seven other genes including the *dif* gene (Steward and Nusslein-Volhard, 1986). *P(PZ)l(2)06270* (mapped in 23F5-6) and *P(lacW)l(3)j5C2* (mapped in 63B7-8) are enhancer trap lines (referred to as *PZ*) which exhibit a strong *lacZ* expression in the larval fat body (personal communication from Berkeley *Drosophila* Genome Project). In order to distinguish homozygous larvae from their heterozygous siblings, second chromosome mutations were balanced either by a *CyOy*⁺ balancer in a *y*, *w* context or by a *CyO*, *Pf*^{w⁺mC} *GFPAct5C.PR* (Reichhart and Ferrandon, 1998) and third chromosome mutations were balanced by the *TM6C*, *Sb*, *Tb* balancer which carries the larval marker *Tubby*. *P{ry⁺i7.2* = *hsFLP*12 (*hsFLP*12; Chou and Perrimon, 1992) and *P{ry⁺i7.2* = *hsFLP*1 (*hsFLP*1; Golic, 1991) were used as *flp*-producing strains. *FRT* strains bearing the *P{ry⁺i7.2* = *neoFRT*} element (hereafter referred to as *FRT* in the text) were as

described by Xu and Rubin (1993). All the chromosomes bearing the *FRT* element and a mutation were produced by standard meiotic recombination crosses, and recombinants were selected by their resistance to G418 (Geneticin, Gibco-BRL). The following *FRT* chromosomes were constructed:

P{ry⁺i7.2 = *neoFRT*40A, b, *cact*^{D13} (*FRT cact*^{D13})
P{ry⁺i7.2 = *neoFRT*40A, *TI*¹¹⁹ (*FRT TI*¹¹⁹)
P{ry⁺i7.2 = *neoFRT*82B, *TI*^{1RXA} (*FRT TI*^{1RXA})
P{ry⁺i7.2 = *neoFRT*82B, *TI*^{9ORE} (*FRT TI*^{9ORE})
P{ry⁺i7.2 = *neoFRT*40A, b, *cact*^{D13}, *TI*¹¹⁹ (*FRT cact*^{D13} *TI*¹¹⁹)
P{ry⁺i7.2 = *neoFRT*40A, *l(2)06270* (*FRT PZ*)
P{ry⁺i7.2 = *neoFRT*82B, *l(3)j5C2* (*FRT PZ*)

The *FRT cact*^{D13} *TI*¹¹⁹ chromosome was obtained by meiotic recombination between a *FRT cact*^{D13} and a *FRT TI*¹¹⁹ chromosome. We examined *cact*^{D13}, *TI*¹¹⁹/*cact*^{D13} mutants and found that the lethality associated with the *cact* null allele *cact*^{D13} is rescued by the loss of one copy of both the *dl* and *dif* genes (P. Manfruell, unpublished data). Two reporter transgenes were recombined on the *y*, *w*, *X* chromosome allowing the analysis of their expression in the same fat body fragment: *drom-GFP* (Ferrandon et al., 1998) for the gene encoding Drosomycin, *dipt-lacZ* (Reichhart et al., 1992) for the gene encoding Dipterin. In experiments shown in Figures 4E and F, 5A and B, 6C–F, a single *drom-GFP* reporter gene inserted on the *X* chromosome (devoided of *dipt-lacZ*) is used. *hs-dif* and *hs-dl* lines were transgenic strains carrying the cDNA of *dif* or *dl* under the control of either the *hsp70* or *hsp83* promoter. *hsp70-dif* and *hsp70-dl* were gifts from Dr R. Steward (Rutgers University, Piscataway, NJ). *hsp83-dif* and *hsp83-dl* are described in Gross et al. (1998). The *act5C>Draf⁺>nuc-lacZ* transgene is described by Struhl and Basler (1993). For complete descriptions of the marker genes and balancer chromosomes used, see Lindsley and Zimm (1992).

Bacterial challenge

Bacterial challenge (also referred to as a septic injury in the text) is obtained by pricking third instar larvae with a thin needle previously dipped into a concentrated bacterial culture of *Escherichia coli* (Gram[−]) and *Micrococcus luteus* (Gram⁺).

RNA preparation and analysis

Total RNA extraction and Northern blotting experiments were performed as in Lemaitre et al. (1995a). The following probes were used: *attacin* cDNA (Asling et al., 1995); *cecropin* A1 cDNA (Kylsten et al., 1990); *dip-tericin* cDNA (Wicker et al., 1990); *drosomycin* cDNA (Fehlbaum et al., 1994) and *rp49* cDNA (a PCR fragment of ~400 bp generated between two oligonucleotides designed after the *rp49* coding sequence; O'Connell and Rosbach, 1984).

Mosaic analysis

The method of heritably activating nuclear *lacZ* expression (FLP-out technique) using the *act5C>Draf⁺>nuc-lacZ* transgene was performed as described by Struhl and Basler (1993). For this procedure, staged embryos carrying both the *hsFLP12* and the *act5C>Draf⁺>nuc-lacZ* insertions were subjected to a single heat shock for 30 min at 37°C at varying times during embryonic development. The emerging larvae were subsequently X-Gal stained (as described in Lemaitre et al., 1995a) to monitor the *nuc-lacZ* staining pattern in fat body and imaginal discs.

Larval mosaic clones were generated as described in Figure 3. In rescue experiments, third instar larvae carrying *hs-dif* or *hs-dl* transgenes were submitted to two heat shocks for 20 min (with a 20 min interval) at 37°C. Bacterial challenge was performed 3 h after heat-shock treatment. Western blots analysis showed that the *DIF* or *DI* proteins were induced in the fat body under these conditions (data not shown). The appropriate use of balancer chromosome allowed the isolation in the offsprings of two genotypes in which the effects of heat-shock and septic injury could be analysed under strictly the same conditions: *hsFLP12/dipt-lacZ*, *drom-GFP*; *FRT TW119/FRT cact*^{D13}; *hs-dif* or *hs-dl/+* and *hsFLP12/dipt-lacZ*, *drom-GFP*; *FRT TW119/FRT cact*^{D13}; *+TM6C* (used as a control since these larvae lack *hs-dif* or *hs-dl*).

Analysis of reporter gene expression

Third instar larvae were immobilized on ice and viewed under epifluorescent illumination (excitation filter 480/40 nm; dichroic filter 505 nm LP; emission filter 510 nm LP) with a Leica MZ12 dissecting scope. Larval fat bodies were dissected in phosphate-buffered saline under the dissecting scope and fluorescence from the *drom-GFP* reporter gene was analysed. The fat body fragment was subsequently stained for

β -galactosidase activity (X-Gal staining). The *dipt-lacZ* transgene showed a cytoplasmic *lacZ* expression whereas the *PZ* insertion used as a cell marker expressed the enzyme in the nucleus. However, in bacteria-challenged third instar larvae, the high expression level of the *dipt-lacZ* reporter gene did not allow the observation of *PZ* marker. GFP images were taken on a 400 ASA Fujicolor film. Images in Figures 2, 3, 4 and 5 were digitized and assembled using Photoshop 4.0 (Adobe). β -galactosidase measurements were performed as in Lemaitre and Coen (1991).

Acknowledgements

The authors are indebted to Emma Langley for anti-DIF antibody, to Konrad Basler (Zurich, Switzerland) for the gift of FLP-out strains, and to Marie Meister, Ranjiv Khush, Dominique Ferrandon and Fotis Kafatos for stimulating discussions. The technical assistance of Reine Klock and Raymonde Syllas is gratefully acknowledged. This work was supported by the CNRS and the University Louis Pasteur of Strasbourg. P.M. wishes to acknowledge the support of the Fondation pour la Recherche Médicale, the Secours des Amis de l'Académie des Sciences and the Training and Mobility Research Program.

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Received November 26, 1998; revised and accepted April 29, 1999