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.

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 (Lemaitre 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 dipterisin (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 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; Lemaitre 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-κB 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 (Lemaitre et al., 1996; Nicolas et al., 1998).
Role of dl and dif in drosomycin gene expression

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 DI, the drosomycin gene (and those of all the other known antimicrobial peptides) remained fully inducible by an immune challenge (Lemaître et al., 1995b). This result indicated that either DI 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 DI in antimicrobial peptide genes. Our previous studies had corroborated our preceding data (Xu et al., 1996). The induction 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 in larvae than previously seen in adults (Lemaître 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. This result indicates that in larvae, the activation of the Tl pathway in Tl^0 mutants is sufficient to trigger a higher drosomycin expression than in adults (Lemaître 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 (Lemaître et al., 1996). The level of drosomycin expression is significantly higher in Tl^0 mutants than that induced by immune challenge in wild-type larvae. In addition, pricking the Tl^0 gain-of-function mutants significantly increases the level of drosomycin expression over that of constitutive expression. The data were analysed by phoshomaging and the results are plotted in Figure 1A.

A striking observation is that the drosomycin gene is strongly expressed in Tl^0 gain-of-function and cact-deficient mutants in the absence of immune challenge. Since our mosaic analysis was carried out with fat body cells, the major site of synthesis of antimicrobial peptides, and their expression is upregulated by immune challenge; the DI 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 DI and DIF bind to similar Rel binding sites that are present in the promoters of all genes encoding antimicrobial peptides (Ips 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 dl and dif 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 DI and DIF activity using this deficiency and the yeast site-specific fip/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 DI and DIF to antimicrobial peptide gene expression, we used dl and dif transgenes under the control of a heat-shock promoter to reintroduce DI 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 (Lemaître 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 dipterin in larvae, requires the Rel proteins DI 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→DI/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, Dipterin, 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^0) 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 phosphomaging and the results are plotted in Figure 1A.
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– and spz– 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. OrR, Oregon R; df+, dl1/dl1; Tl+, Tl632/TlRXA; spz+, spz/wspz; imd, imd/imd; spz, spz/imszp; TlD, Tl10B/1; cact, cactA4/cactD13.

In contrast to the drosomycin gene, the genes encoding the antibacterial peptides Diptericin, Cecropin and Attacin are not constitutively expressed in TlD 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
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 (Lemaitre 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 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 diptericin–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 dipterinc 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, cactD13. 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 dipt–lacZ (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 cactD13 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 cactD13/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 cactD13 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 dipterinc reporter gene in the absence of immune challenge (red dotted lines in Figure 4D).

We further analysed the expression of the drosomycin and the dipterinc genes in fat bodies of larvae containing cells homozygous for Tl null allele (TlQRE). 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 dipterinc 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 constitutively expressed in any tissue other than the fat body cell. Additionally, the mosaic cells deficient for cact do not express the dipterinc reporter gene in unchallenged larvae homozygous for Tl null mutations. (A–D) Mosaics for a cact null allele, cactD13. Mitotic recombination was induced in hsFLP12/dipt–lacZ, drom–GFP; FRT cactD13/FRT l(2)06270 larvae. (A) In the absence of immune challenge, strong fluorescence signals indicating constitutive drom–GFP expression appear 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 cactD13 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, TlQRE. Mitotic recombination was induced in hsFLP12/drom–GFP; FRT TlQRE/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 TlQRE/FRT 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 diptericin

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)TW19 is a small deficiency (hereafter referred to as TW19) 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 TW19 deficiency is embryonic lethal and we first determined if it would be possible to generate viable TW19 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 TW19/FRT l(2)06270 with that in male larvae of genotype +/++; FRT TW19/FRT l(2)06270, which differ by the absence of a heat-shock inducible fpl 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 TW19 deletion. We did note, however, that the TW19 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 TW19. Importantly, we observed that homozygous TW19 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 homzygous TW19 clones by using the previously described FRT l(2)06270 cell marker. In these larvae, all cells express lacZ, except for those homozygous for TW19, which lack the PZ insertion as a result of the fpl/FRT-mediated recombination (Figure 5A). This mosaic expression of the drom–GFP reporter gene after immune challenge in FRT TW19 mosaic was highly reproducible allowing for the unambiguous identification of TW19 homozygous cells.

To facilitate the selection of mosaic larvae in subsequent experiments, we replaced the FRT PZ chromosome by a FRT cacD13 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 TW19 homozygous cells (detected by the lack of drom–GFP expression after immune challenge) in proximity to the cacD13 mosaic cells (a typical picture is shown in Figure 5C).
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 Dl nor the DIF proteins are strictly required for the induction of dipterin. 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 dipterin 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, dl and dif 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 TP 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 (TP-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<sup>P13</sup> 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 *diptericin* (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 DI 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 DI protein is able to trans-activate 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 (Engstöm 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: DI (Steward, 1987; Reichhart et al., 1993) (plus a splice isoform, DI-B; Gross et al., 1999), DIF (Ip et al., 1993) and Relish (Dushay et al., 1996). *In vitro* studies have shown that DI and DIF can transactivate some of the antimicrobial peptide genes in blood cell lines (Petersen et al., 1995; Gross et al., 1996). 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 these cells.

**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*, *pll* 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 adepithelial 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 (*diptericin*) 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. Manfruelli unpublished data). Northern blot analysis, furthermore, indicates that the 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 DI 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, DI 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 DI have somewhat different transactivating capabilities and do not bind κB-motifs present in the upstream regions of the genes encoding Cecropin and Diptericin with the same affinities (Petersen et al., 1995; Gross et al., 1996). DIF and DI 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 DI-induced transcription of this gene.
inducibility of the \textit{drosomycin} gene in TI pathway mutants is less dramatically affected in larvae than in adults. This suggests that another regulatory cascade might partially substitute for the TI pathway in controlling \textit{drosomycin} in larval fat body. \textit{Drosophila} contains several TI-like receptors (Mitcham \textit{et al}., 1996), including 18-wheeler, which is reportedly involved in the control of \textit{attacin} and, to a lesser extent, \textit{ecropin} induction in larvae (Williams \textit{et al}., 1997). However, 18-wheeler mutations do not seem to affect \textit{drosomycin} expression (E.Eldon, personal communication; P.Manfruelli, unpublished data). The possible contribution of these receptors to the humoral immune response, and namely to the regulation of the \textit{drosomycin} gene, awaits further investigation.

Finally, we have noted that in larvae, as in adults, the inducibility of the \textit{drosomycin} gene is slightly reduced in \textit{imd} mutants. This result, in conjunction with studies on \textit{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 \textit{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 TI signaling cascade regulates numerous functions in various tissues at several developmental stages: dorsoventral axis formation (reviewed in Belvin and Anderson, 1996), proliferation of blood cells (Qiu \textit{et al}., 1998), muscle cell attachment (Halfon \textit{et al}., 1995), axon guidance (Rose \textit{et al}., 1997) and larval size (Letou et \textit{al}., 1991). Through our \textit{flp/FRT} approach, we have shown here that the TI and \textit{cact} genes function cell-autonomously to regulate the antifungal response in the larval fat body. This result definitively proves that the \textit{drosomycin} gene expression is directly regulated by the TI pathway in the fat body cells.

**The TI pathway controls the expression of the \textit{drosomycin} gene in larvae via the DIF and DL proteins**

An interesting result of this study is the observation that the constitutive expression of the \textit{drosomycin} gene in \textit{TI} \textit{gain-of-function} mutants is abolished in fat body cells deficient for the \textit{diff} and \textit{dl} genes. This result clearly shows that the TI function in the immune response is mediated by DL and DIF. Similarly, the absence of constitutive expression of the \textit{drosomycin} gene in \textit{cact}, \textit{TW19} clones demonstrates that DIF and DI are actually the Rel proteins sequestered by Cact that control \textit{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 \textit{in vitro} (Kidd, 1992; Leiming \textit{et al}., 1995; Tatei and Levine, 1995; Govind \textit{et al}., 1996). Under \textit{in vitro} conditions, DI and DIF can form heterodimers (Gross \textit{et al}., 1996). However, our rescue experiments clearly show that DIF can function in the absence of DL to regulate \textit{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 \textit{dl} in \textit{cact}, \textit{TW19} cell clones (which lack both Cact and DIF) in the absence of a septic injury leads to the transcription of the \textit{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 TI receptor whereas under the same conditions, DL 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 \textit{TI} \textit{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 \textit{TW19} deficiency. This indicates that in contrast to \textit{drosomycin}, \textit{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 (Lemaire \textit{et al}., 1996), that \textit{diptericin} expression is controlled by mechanisms largely different from those that regulate \textit{drosomycin} expression. The promoter of the \textit{diptericin} gene contains two identical \textit{kB}-sites which are mandatory for the expression of this gene (Kappler \textit{et al}., 1993; Meister \textit{et al}., 1994). Earlier studies from this laboratory have shown that the overexpression of \textit{dl} in cultured cell lines can transactivate a reporter gene placed under the control of eight \textit{diptericin} \textit{kB}-sites (Reichhart \textit{et al}., 1993). This result suggested that DI can play a role in the regulation of \textit{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 \textit{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 (Lemaire \textit{et al}., 1997).

**A clonal analysis of the humoral antimicrobial response**

The present report is the first study making use of the \textit{flp/FRT} approach in larval fat body cells of \textit{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 \textit{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 \textit{serpent} during embryogenesis (Riechmann \textit{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 dipterin 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 dipterin 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 genetic discrepancies 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 analysis of the molecular basis of 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 X chromosome. The fusion gene contains 1.88 kb Xhol–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 and cact, cact10, Tl22, Tl, Tl-KS, Tl10Bp, Tl-20, spätzli and imd mutant lines have been described elsewhere (Anderson and Nusslein-Volhard, 1984; Anderson et al., 1985; Lemaire et al., 1995a,b). Mutants in Tl have been reported to exhibit significant lethality during the larval stage (Gertulla et al., 1988). To obtain Tl larvae and adults, we used two thermosensitive alleles of Tl (Tl(T2a5) and Tl(F25)), which exhibit a strong phenotype only when raised at 29°C (Gertulla et al., 1988). Tl-deficient mutants were reared at 18°C and shifted to 29°C at the second instar. Tl10Bp is a null embryonic lethal allele of Tl. cact10 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)71W119, (TW119) 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]/(2)02670 (mapped in 23F5-6) and P[lacW][WII3]5SC2 (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 homoygous larvae from their heterozygous siblings, second chromosome mutations were balanced either by a CyO1 balancer in a y, w, sectype or by a CyO2, P[w+; P[w+; GFPalpha5CP8] (Reichhart and Ferrandon, 1998) and third chromosome mutations were balanced by the TM6C, Sh, Tb balancer which carries the larval marker Tubby, P[+]/+; hsFLP12 (hsFLP12; Chou and Perrimon, 1992) and P[+]/+; hsFLP11 (hsFLP11; Golic, 1991) were used as fly-producing strains. FRT strains bearing the P[+]/+; 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:

\[ P[y^{+}; \text{neoFRT}]/40A, b, cact10 (FRT cact10) \]

\[ P[y^{+}; \text{neoFRT}]/40A, TW119 (FRT TW119) \]

\[ P[y^{+}; \text{neoFRT}]/40A, Tl22 (FRT Tl22) \]

\[ P[y^{+}; \text{neoFRT}]/2B, Tl-KS (FRT Tl-KS) \]

\[ P[y^{+}; \text{neoFRT}]/2B, Tl10Bp (FRT Tl10Bp) \]

\[ P[y^{+}; \text{neoFRT}]/40A, b, cact10 (FRT cact10) \]

\[ P[y^{+}; \text{neoFRT}]/40A, Tl22 (FRT Tl22) \]

\[ P[y^{+}; \text{neoFRT}]/2B, Tl-KS (FRT Tl-KS) \]

\[ P[y^{+}; \text{neoFRT}]/2B, Tl10Bp (FRT Tl10Bp) \]

\[ P[y^{+}; \text{neoFRT}]/2B, Tl-KS (FRT Tl-KS) \]

\[ P[y^{+}; \text{neoFRT}]/2B, Tl10Bp (FRT Tl10Bp) \]

\[ P[y^{+}; \text{neoFRT}]/2B, Tl-KS (FRT Tl-KS) \]

\[ P[y^{+}; \text{neoFRT}]/2B, Tl10Bp (FRT Tl10Bp) \]

\[ P[y^{+}; \text{neoFRT}]/2B, Tl-KS (FRT Tl-KS) \]

\[ P[y^{+}; \text{neoFRT}]/2B, Tl10Bp (FRT Tl10Bp) \]

The FRT cact10 TW119 chromosome was obtained by meiotic recombination between a FRT cact10 and a FRT TW119 chromosome. We examined cact10, TW119/cact10 mutants and found that the lethality associated with the cact null allele cact10 is rescued by the loss of one copy of both the dl and dif genes (P.Manfrelli, 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 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 hsps3 promoter. hsp70-dif and hsp70-dl were gifts from Dr. R.Steward (Rutgers University, Piscataway, NJ). hsp83-df 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 Lindsay and Zimm (1992).

**Bacterial challenge**

Bacterial challenge (also referred to as a septic injury in the text) is obtained by prick ing 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 Lemaire et al. (1995a). The following probes were used: attacin cDNA (Asling et al., 1995); cecropin A1 cDNA (Kylsten et al., 1990); dipterin 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 Lemaire 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 balance 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 cact10, +/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 cellarker 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 Lemaire and Coen (1991).

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