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Functional analysis and regulation of nuclear import of *dorsal* during the immune response in *Drosophila*

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In addition to its function in embryonic development, the NF-κB/rel-related gene *dorsal* (*dl*) of *Drosophila* is expressed in larval and adult fat body where its RNA expression is enhanced upon injury. Injury also leads to a rapid nuclear translocation of dl from the cytoplasm in fat body cells. Here we present data which strongly suggest that the nuclear localization of dl during the immune response is controlled by the Toll signaling pathway, comprising gene products that participate in the intracellular part of the embryonic dorsoventral pathway. We also report that in mutants such as *Toll* or *cactus*, which exhibit melanotic tumor phenotypes, dl is constitutively nuclear. Together, these results point to a potential link between the Toll signaling pathway and melanotic tumor induction. Although dl has been shown previously to bind to κB-related motifs within the promoter of the antibacterial peptide coding gene diptericin, we find that injury-induced expression of diptericin can occur in the absence of dl. Furthermore, the melanotic tumor phenotype of *Toll* and *cactus* is not dl dependent. These data underline the complexity of the *Drosophila* immune response. Finally, we observed that like other rel proteins, *dl* can control the level of its own transcription.

Key words: antibacterial peptides/dorsal/insect immunity/rel/NF-κB/Toll

Introduction

Like other endopterygote insects, *Drosophila* responds to injury or bacterial challenge by the rapid and transient synthesis of a battery of potent antibacterial peptides (reviewed in Hoffmann *et al.*, 1993; Hultmark, 1993; Cociancich *et al.*, 1994). This innate, non-adaptive response of *Drosophila* shares many of the characteristics of the mammalian acute-phase response (reviewed in Hoffmann *et al.*, 1993; Hultmark, 1993). To date, seven genes encoding bacteria-inducible antibacterial peptides of *Drosophila* have been cloned. These include four cecropins (Kylsten *et al.*, 1990; Tryselius *et al.*, 1992), diptericin (Reichhart *et al.*, 1992), defensin (Dimarcq *et al.*, 1994) and drosocin (M.Charlet, personal communication). They are all expressed in the fat body, the functional homolog of the mammalian liver, and in some blood cells. Remarkably, the upstream regions of all these genes contain sequence motifs related to the κB motif, which is the binding site for the vertebrate transcription factor NF-κB (Sun *et al.*, 1991; Reichhart *et al.*, 1992; Engström *et al.*, 1993).

The diptericin gene, our model of investigation, contains two κB-related motifs in the upstream region. They are contiguous to, or partially overlap with, sequence motifs homologous to mammalian response elements which confer interleukin (IL)-6 and interferon inducibility to the acute-phase response genes (MacDonald *et al.*, 1990; Isshiki *et al.*, 1991; Reichhart *et al.*, 1992; Georgel *et al.*, 1993, and references therein). The functional relevance of the κB-related motifs in the immune inducibility of the diptericin gene was demonstrated by transgenic methods: wild-type promoter sequences were fully inducible, whereas mutations within the κB motifs abolished this response (Kappler *et al.*, 1993). Similar experiments have independently shown that a κB-related motif confers immune inducibility on cecropin genes in *Drosophila* (Engström *et al.*, 1993).

The transcription factor NF-κB is a heterodimer of the p50 and p65 (relA) subunits, each of which is related to the products of the proto-oncogene *c-rel* and the *Drosophila* morphogen *dorsal* (*dl*; Steward, 1987). These transcription factors belong to the Rel family of proteins (reviewed in Baeuerle and Baltimore, 1991; Schmitz *et al.*, 1991). Additional members have been reported from vertebrates, and it is now established that NF-κB can consist of homodimers or heterodimers of any of the subunits (reviewed in Grilli *et al.*, 1993). In uninduced cells, NF-κB is sequestered in the cytoplasm by association with an inhibitor called I-κB, which is dissociated from NF-κB by a variety of inducers including cytokines, mitogens and bacterial lipopolysaccharide. The mechanism by which cytokines such as IL-1 or tumor necrosis factor (TNF)-α cause dissociation of the NF-κB complex is not clear, although there is evidence that phosphorylation of I-κB abolishes its inhibitory activity (Shirakawa and Mizel, 1989; Gosh and Baltimore, 1990; Kerr *et al.*, 1991). Once activated, NF-κB controls the expression of numerous genes, including those involved in immune or inflammatory responses.

In *Drosophila*, the Rel protein dl acts as the morphogen in patterning the embryonic dorsoventral axis (reviewed in Govind and Steward, 1991; Steward and Govind, 1993). The dl protein is initially uniformly distributed in the egg cytoplasm. Prior to gastrulation, 11 genes function to direct its spatially regulated nuclear import. This signaling pathway is initiated during oogenesis and culminates in a

gradient of nuclear dl protein, with peak concentrations in ventral nuclei and progressively lower levels in lateral and dorsal nuclei (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Steward, 1989, and references therein). An inactivating mutation in *cactus* (*cact*) results in ventralized embryos which display nuclear localization of dl in both dorsal and ventral regions (Roth *et al.*, 1989, 1991; Steward, 1989). Loss of function in any of the 10 other genes results in dorsalized embryos (Nüsslein-Volhard, 1979; Anderson and Nüsslein-Volhard, 1984, 1986; Schüpbach and Wieschaus, 1989) in which all dl protein remains cytoplasmic. These genes were placed in a pathway using different approaches. The last component of this pathway, dl, is complexed in the cytoplasm by the product of the *cact* gene, a structural homolog of I-κB (Geisler *et al.*, 1992; Kidd, 1992; Whalen and Steward, 1993). Dissociation of cact from dl is thought to be induced by a Toll (Tl)-dependent signal, Tl being the receptor residing in the plasma membrane of the syncytial blastoderm embryo (Hashimoto *et al.*, 1988, 1991). This signal is transmitted through the cytoplasmic functions of the protein kinase pelle (Shelton and Wasserman, 1993) and the protein tube (Letsou *et al.*, 1991), to promote the nuclear translocation of dl. In wild-type embryos, the gradient of dl nuclear localization controls the transcription of downstream genes which, in turn, are involved in regional patterning along the dorsoventral axis (reviewed in Ferguson and Anderson, 1991).

In its intracellular domain, Tl shares sequence similarity with the intracellular domain of the IL-1 receptor of mammalian lymphocytes (Hashimoto *et al.*, 1988; Gay and Keith, 1991; Schneider *et al.*, 1991). While engaged in unrelated processes of insect pattern formation and vertebrate immunity, the striking structural and functional similarities within the NF-κB/dl systems have led to the idea that this intracellular part of the signaling pathway is conserved during evolution (Schneider *et al.*, 1991; Shelton and Wasserman, 1993; reviewed in Wasserman, 1993).

Two independent reports (Ip *et al.*, 1993; Reichhart *et al.*, 1993) extend this idea. A new *Drosophila* Rel protein Dif (for dl-related immune factor; Ip *et al.*, 1993), not functional during early embryogenesis, is expressed in the larval fat body. In wild-type larvae, it is primarily cytoplasmic. However, upon injury or in genetic backgrounds (e.g. Tl^{D}) in which the dl pathway is signal-independently activated, Dif is nuclear. *In vitro* experiments show that Dif binds to the κB motif within the cecropin A1 gene, and Ip *et al.* (1993) proposed that in the larva Dif mediates an immune response akin to the mammalian acute-phase response.

It turns out that dl, whose early function is well established, is similar to Dif in that it is also expressed in fat bodies (Reichhart *et al.*, 1993). An essential zygotic function for *dl* is not predicted from its genetics since a complete loss of function of *dl* still results in viable animals (Nüsslein-Volhard, 1979). Like Dif, dl is normally localized in the cytoplasm of the fat body and is rapidly imported to the nucleus upon bacterial challenge (Reichhart *et al.*, 1993). dl binds to κB-related motifs *in vitro*; in transfection experiments with the immune-responsive tumorous blood cell line *mbn-2* (Gateff, 1978), dl was able to transactivate sequence-specifically a reporter gene under the control of κB-related motifs (Reichhart *et al.*, 1993).

Given that (i) immune genes have functional κB-related motifs within their promoters, (ii) mutations in *cact* and *Tl* result in a melanotic tumor phenotype which is considered to be a spontaneous immune-like response (Gertulla *et al.*, 1988; Roth *et al.*, 1991), and (iii) *dl* expression and activity are affected by immune challenge (Reichhart *et al.*, 1993), we questioned if components of the embryonic dorsoventral pathway play a role in the injury-induced relocalization of dl.

Here we present evidence which strongly suggests that in the fat body, the control of nuclear localization of the dl protein during the immune response is controlled by the intracellular embryonic dorsoventral pathway. This nuclear uptake of dl occurs in the absence of challenge in mutants exhibiting melanotic tumors, which suggests that the Tl signaling pathway and the process of melanotic tumor induction are somehow linked. We have found that dl alone does not control the expression of antibacterial peptide genes as these genes are inducible in its absence. Our studies also uncover a new aspect of regulation of *dl*: like other Rel proteins, *dl* is involved in its own expression.

Results

Nuclear import of dl is controlled by proteins of the intracellular dorsoventral pathway

We have analyzed the nuclear translocation of dl by using a monoclonal antibody directed against its unique C-terminal domain (Whalen and Steward, 1993), which is highly divergent from its counterpart in Dif (Ip *et al.*, 1993). As reported previously (Reichhart *et al.*, 1993), dl is predominantly cytoplasmic in fat bodies derived from unchallenged wild-type animals. The observed staining was somewhat variable from one larva to the other (Figure 1A and B), but most of the fat bodies exhibited a very low signal (Figure 1A). When wandering third instar larvae were challenged by needle, with or without bacteria, the nuclear translocation of dl was clearly visible within 15–30 min (Figure 1C). This nuclear staining persisted for several hours and was even observed from early pupal samples (data not shown). Injury sets in a melanization reaction which appears around the wound site in <15 min.

All cells of the dissected fat body showed uniform dl staining, unlike the situation reported for Dif where nuclear staining in response to infection or injury is somewhat erratic and is detected typically in only about one third of the total fat body cells (Ip *et al.*, 1993). Immune-unrelated stress, such as water immersion or heat-shock, did not induce nuclear translocation of dl (data not shown). As expected, no staining was observed with this monoclonal antibody in mutant dl^{1} larvae (Figure 1D) from which the dl protein was absent (Isoda *et al.*, 1992).

We wished to determine whether in fat body cells components of the embryonic dorsoventral pathway are also functional in controlling the nuclear import of dl during the immune response. Our experimental approach was based on the study of the subcellular localization of dl in unchallenged and challenged mutant larvae. For the sake of simplicity, we present our staining results in three parts. First we present data from animals mutant in those

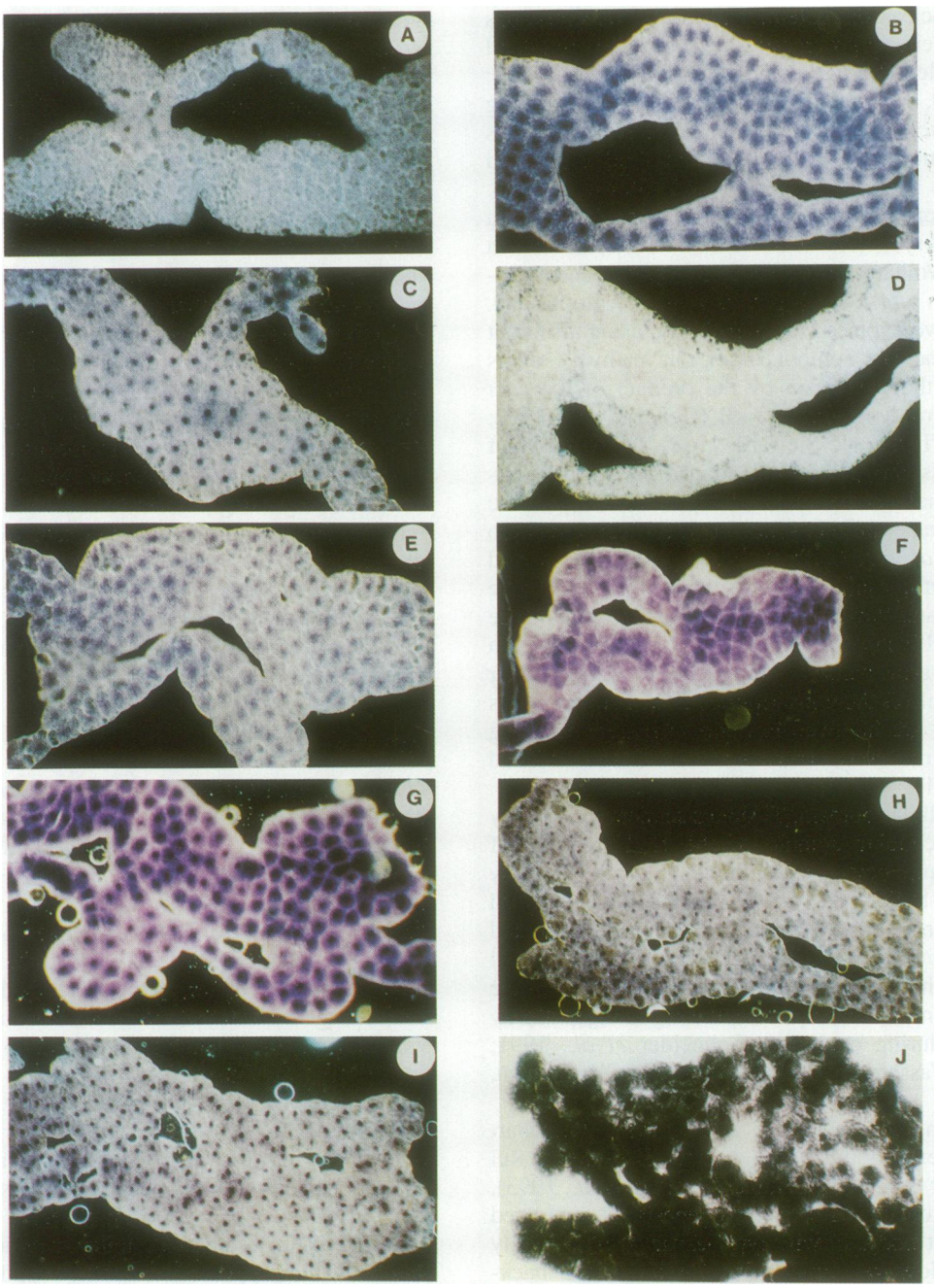

Fig. 1. Immunolocalization of dorsal in *Drosophila* fat body. Fat bodies were dissected and treated with a monoclonal antibody directed against the C-terminal domain of the dl protein. The immunolocalization was visualized with an alkaline phosphatase reaction (AP-conjugated secondary antibody). Photographs (except for J) were taken in dark field which allows a better visualization of cytoplasmic staining; ×50 magnification. Immunolocalization experiments with fat bodies from control and mutant larvae were always run in parallel to allow comparisons. However, the photographs shown in this figure were taken from different experimental series and are representative of the variable stainings. (**A** and **B**) Fat bodies dissected from wild-type unchallenged larvae. All fat body cells show a staining predominantly restricted to the cytoplasm. Staining was variable from one fat body to another; (A) and (B) represent the range of the signal intensity. (**C**) Fat body dissected from a 2 h-challenged wild-type larva. Staining is predominantly nuclear. (**D**) Fat body dissected from a 2 h-challenged mutant dl^{1}/dl^{1} larva. No staining was observed in this negative control. (**E–G**) Fat bodies from challenged *pll*, *tub* and *Tl* mutants: these photographs are representative of the variable stainings. (E and F) Fat body from a 2 h-challenged pll^{078}/pll^{rm8} and a tub^{238}/tub^{118} mutant larva, respectively. The staining is predominantly cytoplasmic. Note that the staining in (F) is stronger than in unchallenged wild-type larvae. (G) Fat body from a 2 h-challenged Tl^{632}/Tl^{9QRE} mutant larva. Tl^{632} is a thermosensitive allele of *Tl*. Tl^{-} larvae were placed at the restrictive temperature (29°C) 16 h before challenge. The staining is both cytoplasmic and nuclear. (**H**) Fat body from an unchallenged $cact^{A2}/cact^{A2}$ larva. The staining is nuclear. (**I**) Fat body from an unchallenged $Tl^{9Q}/+$ larva. Nuclear staining is observed. (**J**) Fat body from an unchallenged $Tl^{10b}/+$ larva. The photograph was taken in bright field which allows a better visualization of the fat body morphology. Note that the cells are rounded and are poorly adherent to one another. Staining is nuclear.

dorsal group genes whose products are required in the perivitelline fluid of the *Drosophila* embryo to generate the Tl ligand (Stein *et al.*, 1991; Stein and Nüsslein-Volhard, 1992; Chasan *et al.*, 1992; Morisato and Anderson, 1994; Schneider *et al.*, 1994; Smith and DeLotto, 1994). These are *gastrulation defective* (*gd*), *snake* (*snk*), *easter* (*ea*) and *spätzle* (*spz*). Next we considered animals mutant in *Tl*, *pelle* (*pll*) and *tube* (*tub*),

Table I. Dorsal localization in various unchallenged and challenged mutant larvae

	Dorsal localization
Unchallenged larvae	
Control (Or^R)	C
$cact^{A2}/cact^{A2}$	N
$Tl^{9Q}/+$	N
$Tl^{10b}/+$	N
Challenged larvae	
Control (Or^R)	N
Control (dl^1)	no protein
tub^{238}/tub^{118}	C*
tub^{238}/tub^{3}	C*
pll^{078}/pll^{rm8}	C*
pll^{74}/pll^{74}	C*
Tl^{9QRE}/Tl^{r444} (29°C)	C*
Tl^{9QRE}/Tl^{632} (29°C)	C*
$Tl^{10b}+$	N
spz^{rm7}/spz^{rm7}	N
spz^{197}/spz^{197}	N
ea^1/ea^1	N
ea^2/ea^2	N
snk^{073}/snk^{073}	N
gd^7/gd^7	N

Immunolocalization assays were performed with control and 90–120 min-challenged wandering third instar larvae. Experiments were carried out at 25°C except for Tl^- mutants. N, the dorsal staining is predominantly nuclear; C, a weak, predominantly cytoplasmic, staining is observed; C*, the dorsal staining is predominantly cytoplasmic but the overall signal is generally higher than in unchallenged wild-type larvae.

the receptor and the two proteins that function within the embryo (Hashimoto *et al.*, 1988; Letsou *et al.*, 1991; Hecht and Anderson, 1993; Shelton and Wasserman, 1993). The effects of all these mutations were tested in challenged larvae. Finally, we looked at animals with loss-of-function mutations in *cact*, as well as those carrying gain-of-function mutations in *Tl* (Tl^D), both of which have similar effects on the nuclear localization of dl in the embryo. These animals were tested in the absence of challenge. The results of all these staining experiments are summarized in Table I.

As indicated in Table I, we have observed that the nuclear translocation of dl induced by injury was not affected significantly by mutations in any of the *gd*, *snk*, *ea* or *spz* genes. This result suggests that these proteins, functional in the extracellular embryonic space, do not control dl nuclear translocation during the immune response. In contrast, when *Tl*, *tub* or *pll* mutant animals were challenged, dl remained predominantly cytoplasmic (e.g. in Figure 1E–G). The overall level of immunostaining of dl in these challenged mutant larvae was higher than in unchallenged wild-type (compare, for example, Figure 1E–G with A and B) or mutant larvae (data not shown). These data indicate that while the level of dl protein increases in response to an immune challenge in these mutant animals, the ability of dl to translocate to the nucleus is markedly reduced.

When we compare our staining results with those observed for the embryo (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Steward, 1989), we find that overall our results are more variable. This is particularly true for the *pll*, *tub* and *Tl* loss-of-function mutants, in which occasionally some dl is seen in the nucleus (clearly visible in Figure 1G). This may be due to the fact that the cells of the fat body respond to the signal in a different way to the embryo. Alternatively, it may be that there is yet another signaling pathway which is required to fully control this nuclear uptake process.

Next, the role of *cact* was studied. The results were clear-cut and are illustrated in Figure 1H which shows a nuclear enrichment of dl in unchallenged *cact* animals. Interestingly, the same result was observed in animals with dominant *Tl* mutations (Tl^{9Q} and Tl^{10b}; Figure 1I and J). The localization of dl was not altered upon challenging these animals (data not shown). Altogether, these results strongly suggest that the intracellular but not the extracellular component of the dorsoventral signaling pathway is involved in injury-induced dl import.

The melanotic tumor phenotype of Tl

Neither *Tl* nor *cact* are strict maternal-effect genes. While null mutations in *Tl* result in partial lethality (Gertulla *et al.*, 1988), strong alleles of *cact* are completely lethal (Roth *et al.*, 1991). Thus, *cact* is essential for one or more zygotic functions. $cact^{A2}$, the allele of *cact* used in our experiments, is the strongest viable allele of *cact* (Roth *et al.*, 1991) and exhibits a significant lethality at the end of the third instar larval stage (data not shown). It is interesting that both Tl^D and viable *cact* mutations not only show a constitutive nuclear localization of dl in the fat body, but also exhibit a melanotic tumor phenotype (Figure 2A; Gertulla *et al.*, 1988; Roth *et al.*, 1991). Since Tl^D, in contrast to *cact*, does not significantly affect the viability, we have restricted our investigation to this mutation.

Melanotic tumors are non-invasive and are generally considered to represent an immune-like response to invasive foreign or altered self substances (for a review see Sparrow, 1978). The melanotic tumor phenotype results from abnormalities in the differentiation of hemocytes. In normal larvae the hemocyte population consists almost entirely of small rounded cells referred to as plasmatocytes (Figure 2B), which in the wild-type differentiate into large flat lamellocytes shortly after puparium formation (Figure 2C; Rizki, 1957). Lamellocytes play a central role in the encapsulation process of foreign bodies or of cell debris derived from tissue remodeling during metamorphosis (Whitten, 1964; Rizki and Rizki, 1978; Watson *et al.*, 1991). To understand the possible role of *Tl* in cellular immunity, we examined the hemocyte populations from Tl^D mutants. Interestingly, we found that in $Tl^{10b}/+$ and $Tl^{9Q}/+$ larvae, up to 50% of all hemocytes were lamellocytes, whereas in the hemolymph of wild-type larvae these lamellocytes were not observed (Figure 2B and C). Similar results have been reported for other melanotic tumor mutants (Sparrow, 1978; Nappi and Carton, 1986).

Tl^D larvae presented an abnormal fat body morphology (Figure 1J; see also Ip *et al.*, 1993); the phenotype was more pronounced with Tl^{10b} than with Tl^{9Q}. The fat body cells appeared rounded and were markedly less adherent to one another than in wild-type larvae.

Constitutive nuclear localization of dl in melanotic tumor mutants

Since dl was nuclear in fat body cells of Tl^D and *cact* animals, and both these mutations resulted in melanotic

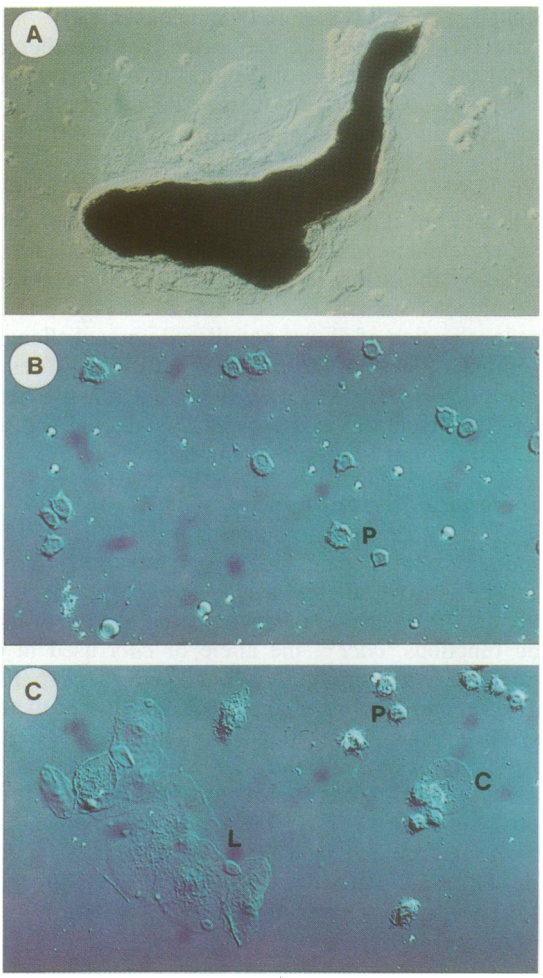

Fig. 2. The melanotic tumor phenotype of dominant *Tl* larvae. (**A**) Phase-contrast view of a Tl^{9Q} melanotic body showing the lamellocyte capsule; ×400 magnification. (**B**) Circulating hemocytes in the hemolymph of a wild-type third instar larva viewed under phase contrast. (**C**) Phase-contrast view of Tl^{9Q} circulating hemocytes including lamellocytes (L), plasmatocytes (P) and crystal cells (C). Abnormal presence of lamellocytes is observed in all the melanotic tumor mutants studied so far.

tumors, we were curious to examine the subcellular distribution of dl in other melanotic tumor mutants that are not known to function in the Tl signaling pathway. $l(2)37Bc^{9}$ and *modulo* (*mod*) are two mutants causing a larval lethality which show a high frequency of melanotic tumors (Wright *et al.*, 1976; Garzino *et al.*, 1992). 1046Bc is a line carrying a *Bc* mutation and exhibiting a thermosensitive tumor phenotype (Rizki *et al.*, 1980; see Materials and methods). We have observed that larvae carrying any of these mutations showed marked nuclear staining of dl in the absence of challenge.

Larvae from the 1046Bc line, reared at the permissive temperature (18°C), do not show melanotic tumors (B.Lemaitre, unpublished data). In these larvae, dl staining was predominantly cytoplasmic (Figure 3A). At 25°C, most of the larvae exhibited melanotic tumors and dl was predominantly nuclear (Figure 3B). This result underlines the correlation between the presence of tumors and the nuclear localization of dl.

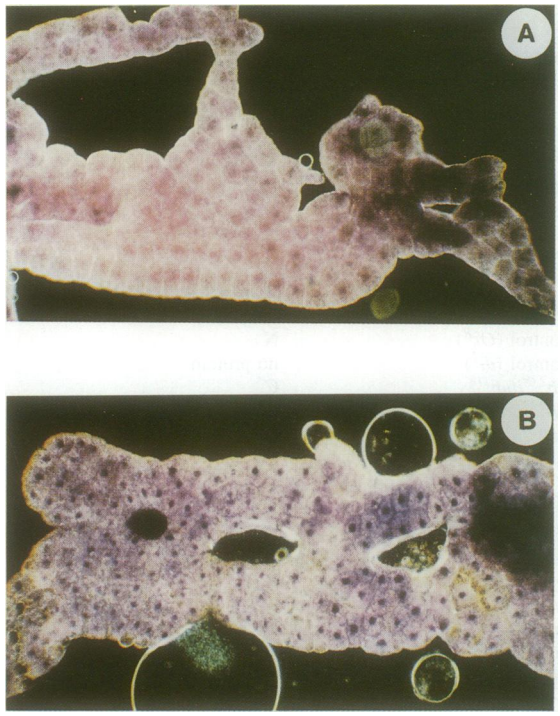

Fig. 3. dl protein is nuclear in melanotic tumor mutants. (**A**) Fat body dissected from an unchallenged mutant larva from the 1046Bc line reared at 18°C. The staining is predominantly cytoplasmic; ×50 magnification. (**B**) Fat body dissected from an unchallenged mutant larva from the 1046Bc line reared at 25°C. Note that a high fraction of the signal is nuclear.

A role for dl in cellular or humoral immunity?

To test if the melanotic tumor phenotype in the *cact* and Tl^{D} background is dl dependent, we generated double mutants. The incidence of tumors was recorded in larvae or in adults, since melanotic tumors persist in the abdominal cavity after metamorphosis. We observed that $cact^{A2},dl^{1}/cact^{A2},dl^{1}$ or $dl^{T}/Df(dl);Tl^{10b}/+$ or $dl^{1}/dl^{1};Tl^{9Q}/+$ animals clearly exhibited melanotic tumors as well as a high proportion of lamellocytes in their hemolymph (data not shown). The $dl^{T}/Df(dl);Tl^{10b}/+$ mutant larvae also exhibited an abnormal fat body morphology. These observations strongly indicate that dl by itself is not essential for melanotic tumor formation in *cact* and Tl^{D} mutants and that lamellocyte differentiation can occur in the absence of dl.

Upon bacterial challenge, the rapid nuclear translocation of dl occurs concurrently with the induction of the diptericin gene (Reichhart *et al.*, 1993; this study). Given the possibility that the diptericin promoter could be a potential target of dl, we considered whether dl plays a role in the induction of diptericin. We studied the induced expression of diptericin in various *dl* mutants.

We first used the transgenic approach. Animals carrying the promoter fusion Dipt2.2−*lacZ* reporter construct reproduce the induction of the endogenous diptericin gene (Reichhart *et al.*, 1992). Flies carrying hypomorphic alleles of *dl* (dl^{D7}, dl^{2}, dl^{SC}, dl^{5}, dl^{PZ}, dl^{U5} and dl^{011}) were crossed with the Dipt2.2−*lacZ* flies. These *dl* alleles code for mutant proteins (Isoda *et al.*, 1992). The results were clear: *in trans* to deficiency *Df(2L)TW119*, none of these alleles affected the inducibility of the Dipt2.2−*lacZ* transgene (data not shown).

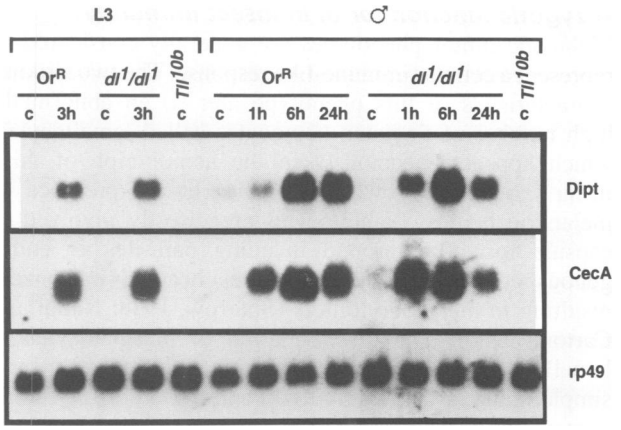

Fig. 4. Transcriptional profiles of diptericin and cecropin A in wild-type and mutant *Drosophila*. Total RNA was extracted at different time intervals (as indicated) after septic injury from wild-type Oregon^R (Or^R) and dl^1/dl^1 mutants. 20 μg samples were fractionated by denaturing 1% agarose–formaldehyde gel electrophoresis, transferred onto a nylon membrane and successively hybridized with a nick-translated diptericin cDNA probe (Dipt), an end-labeled oligonucleotide probe complementary to cecropins A1 and A2 (CecA) transcripts and a nick-translated rp49 cDNA probe. c, control unchallenged animals; L3, wandering third instar larvae.

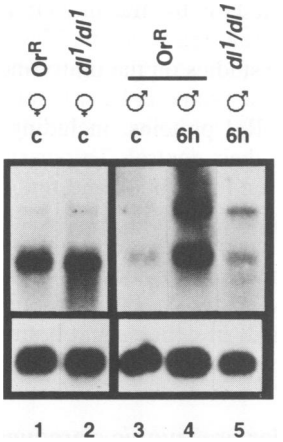

Fig. 5. Induction of *dl* transcription in wild-type and mutant *Drosophila*. Poly(A)-enriched RNA was extracted from control (c) or 6 h bacteria-challenged wild-type Oregon^R (Or^R) and dl^1/dl^1 mutants. 5 μg samples were fractionated on a 1% agarose–formaldehyde gel, transferred onto a nylon membrane and successively hybridized with a nick-translated *dl* cDNA probe and an rp49 cDNA probe (lower part). The canonical *dl* transcript of 2.8 kb is observed with a larger transcript of 4.4 kb which has already been reported by Reichhart *et al.* (1993).

We then performed a Northern analysis with a null allele of *dl*. Total RNA from control or bacteria-challenged wild-type or dl^1 larvae and adults was probed with a diptericin cDNA. As is apparent in Figure 4, in the dl^1 mutants the diptericin gene was fully induced. The variability in intensity of the signal of the transcripts in these experiments probably reflects the differences in injuries inflicted on the animals. These assays were also performed for the cecropin A genes, and similar results were observed (Figure 4).

To determine if there is a correlation between induction of diptericin and constitutive nuclear localization of dl, we tested the expression of the Dipt2.2–*lacZ* transgene in unchallenged *cact* and Tl^D mutant larvae. In Tl^{10b}/+ or $cact^{A2}/cact^{A2}$ mutants, diptericin was not induced when assayed either with the Dipt2.2–*lacZ* transgene (data not shown) or by Northern analysis (see Figure 4 for Tl^{10b} mutants). The cecropin A genes were also unaffected by these mutations. These results suggest either that dl is not involved in the formation of melanotic tumors of *Tl/cact* mutants nor in the induction of diptericin/cecropin genes, or that dl acts in concert with other proteins to affect cellular and/or humoral immunity.

Evidence for a role of dl in its own expression

Although dl does not appear to be a key player in the induction of anti-bacterial peptide genes, we wanted to determine if it could control the expression of its own gene. A number of studies (reviewed in Gilmore, 1994) point to different ways in which *rel* genes are auto-regulated. By Northern analysis, Reichhart *et al.* (1993) have shown that in adult males the amount of *dl* transcripts is faint, but detectable; it is markedly enhanced upon bacterial challenge. This induction is not as striking in larvae since the uninduced levels are higher than those in males. We therefore focused on male adults. Our experiments were based on high-stringency hybridization with *dl* cDNA of Northern blots prepared with poly(A)-enriched RNA extracted from wild-type and mutant *Drosophila*. Figure 5 (lanes 1 and 2) shows that the level of *dl* transcripts in homozygous dl^1 females is similar to that observed in wild-type females, suggesting that in this amorphic mutation the level of *dl* transcripts remains unaltered. We think that this mutation perhaps affects a step during the biosynthesis of a functional dl protein. We therefore used dl^1 to enquire if the transcription of *dl* was affected in the absence of dl protein.

To our surprise, we found that the level of transcripts in challenged dl^1 males is markedly lower than in corresponding challenged wild-type males. This is clearly seen in Figure 5 (lanes 4 and 5), which compares the level of *dl* transcripts from challenged wild-type and dl^1 male adults. Although we cannot exclude the possibility that dl could affect the stability of its own mRNA, this experiment lends strong support to the idea that the dl protein is required for full inducibility of the transcription of the *dl* gene in response to immune challenge.

Discussion

It is now known that both the anteroposterior and the dorsoventral axes of the *Drosophila* oocyte and embryo are established by processes involving signal transduction. Many of the genes essential for axis formation code for molecules that act in well-conserved pathways such as the epidermal growth factor receptor/*ras* pathway, the serine protease cascade, the neurogenic signaling pathway and the dl/Rel pathway (reviewed in Steward and Govind, 1993; Ruohola-Baker *et al.*, 1994). Most of these signaling pathways are conserved over a considerable evolutionary distance. In particular, the intra-embryonic dl pathway in *Drosophila* shares striking similarities with the pathway which regulates the activity of Rel proteins during the immune response in vertebrates (Wasserman, 1993). dl exerts its mophogenetic effects in the embryo through its selective nuclear import, tightly controlled by a signaling

pathway in which Tl is the transmembrane receptor. This pathway is often referred to as the Tl signaling pathway.

Our molecular studies on the injury-induced expression of diptericin, whose promoter has κB-related binding sites, led us to ask if Rel proteins, including dl, were in any way involved in their control. To our surprise, we found that dl is expressed in fat body and that this expression is appreciably enhanced upon injury (Reichhart *et al.*, 1993). We have followed up on these observations to ask if the injury-induced nuclear translocation of dl is controlled by the Tl signaling pathway. Furthermore, we explored a possible role for dl in cellular and humoral immunity, as well as in the expression of *dl* itself.

The intracellular embryonic dorsoventral pathway and nuclear dl localization in the fat body

Our results of immunostaining of fat body from various mutant larvae led to the following conclusions: (i) activities needed to produce an active ligand for Tl in the perivitelline fluid (*gd*, *snk*, *ea* and *spz*) do not seem essential; (ii) Tl, pll and tub promote injury-induced nuclear uptake of dl; and (iii) cact exerts an inhibitory effect on this process. It is interesting to note that mutations in any of the four genes involved in the intracellular dorsoventral pathway (*Tl*, *pll*, *tub* and *cact*) led to a zygotic phenotype. Strong mutations in *cact* caused completely penetrant larval/pupal lethality (Roth *et al.*, 1991), whereas null mutations in *Tl*, *pll* or *tub* resulted in a significant decrease in viability (Gertulla *et al.*, 1988; Hecht and Anderson, 1993). The causes of this lethality remain to be established, and we do not know whether this lethality is related to the immune response.

Although we cannot exclude that our observations could be due to indirect effects of the mutations which were tested, our data suggest that the regulatory cassette that controls nuclear uptake of dl in the embryo, as well as rel/NF-κB in vertebrate cells, also controls the nuclear uptake of dl in the fat body in response to immune challenge. These results lend additional credence to the idea that this cascade is an ancient signaling pathway involved in first-line host defense (Ip *et al.*, 1993). That Rel-related Dif also shows nuclear localization either upon immune challenge or in Tl^D background suggests that in the fat body, nuclear translocation of both Dif and dl are controlled by at least one common regulatory element.

The proteolytic cascade leading to the production of the Tl ligand (Chasan *et al.*, 1992; Stein and Nüsslein-Volhard, 1992; Morisato and Anderson, 1994; Schneider *et al.*, 1994; Smith and DeLotto, 1994) is apparently not involved in the selective nuclear uptake of dl. This is illustrated by observations where larvae mutant in *gd*, *snk*, *ea* or *spz* exhibit injury-induced nuclear uptake of dl, much like wild-type larvae. We do not understand the nature of the stimulus or that of the ligand that induces fat body cells to respond to challenge. We may speculate that it is derived from one of the proteolytic cascades triggered by immune challenge, such as the coagulation or the prophenoloxidase cascade (discussed in Hultmark, 1993; Sugumaran and Kanost, 1993). Alternatively, molecules such as lipopolysaccharides might bind to membrane receptors on the fat body cells and directly elicit an immune response.

A zygotic function for dl in insect immunity?

Melanotic tumor phenotypes are generally considered to represent a cellular immune-like response. The two striking characteristics of this phenotype are: (i) an abnormally high number of flattened hemocytes called lamellocytes, which appear precociously in the hemolymph of third instar larvae (Sparrow, 1978), and (ii) the presence of melanotic bodies. Lamellocytes are directly involved in capsule formation around invading parasites or endogenous cell debris; these capsules become melanized, resulting in melanotic tumors (Sparrow, 1978; Nappi and Carton, 1986). The differentiation of plasmatocytes in lamellocytes can be induced by infection and even by a simple injury, as shown by Rizki and Rizki (1992). In this context, it is interesting to recall our observations that ventralizing alleles of *Tl* and *cact*, in which dl is constitutively nuclear in the embryo or in the fat body, exhibit melanotic tumors. Conversely, in at least three melanotic tumor mutants a marked dl signal is observed in the nuclei. These data suggest that the Tl signaling pathway is involved in the formation of melanotic tumors and possibly in lamellocyte differentiation. Experiments are underway to probe this idea.

We tested whether a causal relationship existed between nuclear dl (e.g. in Tl^D or *cact* mutants) and melanotic tumor formation. Our results with double mutants clearly showed that dl is not required for either lamellocyte differentiation or the induction of the melanotic tumor phenotype. Furthermore, the abnormal fat body morphology observed in Tl^{10b}/+ mutants was also observed in the double mutants, indicating that this is also independent of a dl function. This result could be explained by assuming that in dominant *Tl* mutants, Dif, which is constitutively nuclear (Ip *et al.*, 1993), induces the melanotic tumor phenotype and lamellocyte differentiation in the absence of dl. We cannot exclude, however, that the melanotic tumor phenotype in Tl^{10b} mutants could be unrelated to the nuclear localization of dl and Dif. An interesting observation in this context is that expression of the mammalian NF-κB subunit p50 in *Drosophila* leads to lamellocyte differentiation and the formation of melanotic tumors (S.Govind, personal communication). This result strongly suggests that this response is induced by a Rel protein.

Our experiments also showed that the bacteria-induced expression of the diptericin gene does not require the activity of dl. Thus, in *dl* null mutants both the diptericin and the cecropin A genes remain inducible. In addition, preliminary results indicate that the diptericin gene (monitored by the expression of the diptericin–*lacZ* reporter gene) remains inducible in Tl^-, pll^- and tub^- third instar larvae (B.Lemaitre, unpublished data). This suggests that a Tl-independent signaling pathway is involved in the induction of genes encoding antibacterial peptides. Furthermore, the nuclear localization of dl is not sufficient for the induction of diptericin, since in Tl^D or in *cact* mutant larvae the diptericin gene is not induced. These results indicate that other transcription factors, and possibly other Rel proteins, are involved in the control of transcription of the diptericin gene. One of the best candidates is the Rel-related protein Dif which can bind the κB-related sites in the cecropin gene promoter (Ip *et al.*, 1993). A plausible hypothesis is that dl and Dif can

heterodimerize in this system and that, in the absence of the dl protein, Dif can act as a homodimer and still regulate the transcription via the κB-related sites. Nevertheless, the situation is certainly more complex since in gain-of-function Tl^{10b} larvae both dl and Dif are translocated into the nucleus and yet neither the diptericin nor the cecropin A genes are transcribed. This indicates that either an additional partner is involved, which is not activated by the Tl cascade, or dl or Dif (or both) must undergo a Tl-independent post-translational modification in addition to their nuclear translocation to become capable of activating immune gene transcription. Our data reveal the complexity of the *Drosophila* immune response. The understanding of the exact role of *dl* will necessitate the study of its interactions with Dif and probably with other transactivators in the fat body and blood cells of *Drosophila*.

Finally we leave open the possibility that dl regulates other functions which have not been considered in our study or affects the induction of antibacterial peptide genes and/or lamellocyte differentiation in a more subtle way.

Autoregulation of dl

Rel/NF-κB family members function as protein complexes to exert transcriptional control on many cellular and viral genes. Moreover, Rel protein complexes themselves undergo regulation in many ways. The structure of the rel-homology domain allows for combinatorial dimerization, subcellular compartmentalization, proteolysis and autoregulatory and post-translational modification (reviewed in Gilmore, 1994). The transcriptional functions of dl during embryogenesis have been studied in some detail (e.g. Jiang and Levine, 1993; reviewed in Steward and Govind, 1993), as has its dimerization/cact interaction and subcellular distribution (Govind *et al.*, 1992; Kidd, 1992; Whalen and Steward, 1993). The occurrence of autoregulation has been documented for the c-*rel* (Hannink and Temin, 1990) and p50 (Ten *et al.*, 1992; Cogswell *et al.*, 1993) genes. Our observations that *dl* transcripts are detectable throughout development, albeit at low levels, and are increased upon immune challenge (Reichhart *et al.*, 1993; this study) prompted us to ask whether dl controls its own expression. Although we cannot definitively rule out that dl affects its own mRNA stability, our Northern analysis with challenged dl^1 mutants strongly suggests that dl protein upregulates the level of *dl* transcription considerably, particularly in adult males. It will be interesting to determine if the *dl* promoter has dl binding sites and if this autoregulation function of dl is mediated via these binding sites. It is also possible that this aspect of regulation occurs via another protein complex whose distribution or function is dl dependent.

Materials and methods

Drosophila stocks

Oregon^R flies were used as a standard wild-type strain. The transgenic strain, Dipt2.2−*lacZ*:1 is a ry^{506} C.S. line carrying a diptericin reporter gene on the X chromosome (Reichhart *et al.*, 1992). The fusion gene contains 2.2 kb of diptericin upstream sequences fused to the bacterial *lacZ* coding region and was inserted into the Carnegie 20 vector (Rubin and Spradling, 1983). The developmental and inducible expression of the Dipt2.2−*lacZ* transgene has been described previously (Reichhart *et al.*, 1992): it is superimposable on that of the resident diptericin gene at the end of the third larval instar.

1046Bc is a line from the Bloomington Center homozygous for the *Bc* (Black cells) mutation. *Bc/Bc* larvae have no phenol oxidase activity in the cell-free hemolymph and fail to darken after injury (Rizki *et al.*, 1980). This line also exhibits a high frequency of tumors at high temperature (B.Lemaitre *et al.*, unpublished data). $l(2)37Bc^9$ is a late larval lethal line associated with melanotic tumors (Wright *et al.*, 1976). *A4-4l8* is a lethal line carrying an amorphic mutation in *modulo* (*mod*) which also produces melanotic tumors (Garzino *et al.*, 1992).

dl^1 is a complete loss-of-function (amorphic) mutation as judged by genetic criteria in the embryo (Nüsslein-Volhard, 1979). In this mutant, the dl protein is absent from embryonic extracts (Roth *et al.*, 1991), as well as in bacteria-challenged adult extracts (Reichhart *et al.*, 1993). $In(2L)dl^T$ (called dl^T in the text) is an inversion with a breakpoint in the *dl* locus (Steward *et al.*, 1984). *Df(2L)TW119* is a small deficiency which deletes *dl* and uncovers the 36C interval (Steward *et al.*, 1984). The other *dl* alleles present various strengths of embryonic mutant phenotypes. Sequence analyses suggest that their mutant phenotypes are caused by a single amino acid change which affects either the N-terminal (dl^{D7}, dl^2, dl^{SC}, dl^5, dl^{PZ}) or C-terminal domains (dl^{U5}, dl^{011}) of the dl protein (Isoda *et al.*, 1992). dl^{D7} is a dominant allele of *dl* (Szabad *et al.*, 1989; Isoda *et al.*, 1992) caused by a single amino acid change in the putative DNA binding domain of the dl protein. Experiments reported in the literature suggest that the protein produced by dl^{D7} mutants forms heterodimers with the wild-type *dl* protein but may be unable to bind DNA (Govind *et al.*, 1992).

$cact^{A2}$ is the strongest viable allele of *cact*. Homozygous $cact^{A2}$ females produce strongly ventralized embryos (Roth *et al.*, 1991). $cact^{A2}/cact^{A2}$ larvae exhibit a melanotic tumor phenotype, as noted previously for zygotic lethal alleles of *cact* (Roth *et al.*, 1991). $cact^{A2},dl^1$ is a recombinant line (kindly given by Christiane Nüsslein-Volhard) carrying both mutations (Roth *et al.*, 1991). To identify homozygous larvae from their heterozygous siblings, *dl* and *cact* lines were constructed which carried a *y*, *w* X chromosome and a $CyOy^+$ balancer. Homozygous mutant larvae could thus be distinguished by their yellowish mouth parts. By ordinary crosses, we constructed *dl* and *cact* mutants carrying the Dipt2.2−*lacZ*:1 element on the X chromosome to study the expression of the diptericin reporter gene in these mutants.

Tl^{10b} *and* Tl^{9Q} are two dominant gain-of-function ventralizing alleles of *Tl* caused by a single amino acid change (Schneider *et al.*, 1991). $Tl^{9Q}/+$ and $Tl^{10b}/+$ females produce strongly ventralized embryos. A melanotic tumor phenotype is also observed in larvae carrying these two mutations (Gertulla *et al.*, 1988). tub^{238}, tub^{118}, tub^3, pll^{078}, pll^{rm8}, Tl^{9QRE}, Tl^{rr444}, Tl^{r632}, spz^{rm7}, spz^{197}, ea^1, ea^2, snk^{073} and gd^7 are described in Anderson and Nüsslein-Volhard (1984, 1986) and Hecht and Anderson (1993). The *tub*, *pll*, *Tl*, *spz*, *ea* and *snk* mutations were balanced by the TM6B balancer which carries the larval marker *Tubby*. Homozygous mutant larvae were distinguished from their siblings as '$Tubby^+$'.

All experiments were performed at 25°C unless otherwise stated. For complete descriptions of the marker genes and balancer chromosomes, see Lindsley and Zimm (1992).

Injury experiments

Injury experiments were performed by pricking third instar larvae or adults with a sodium nitrite sharpened tungsten needle. This treatment induced the nuclear translocation of the dl protein in the fat body of wild-type *Drosophila*. For bacterial challenge, the needle was previously dipped into a concentrated bacterial culture of *Escherichia coli* and *Micrococcus luteus*.

β-Galactosidase localization

The fat bodies were fixed for 4 min in PBS, pH 7.5, containing 1% glutaraldehyde and 1 mM $MgCl_2$, washed in PBS and immersed in 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 3.5 mM $K_4Fe(CN)_6$, 3.5 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$, 150 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, and incubated for 4 h at 37°C (Hiromi *et al.*, 1985). They were mounted in glycerol.

Immunolocalization experiments

The accuracy of the immunolocalization assay described below was checked by staining fat body from a P-*lacZ* insertion line known to express β-galactosidase in the fat body (line L44, kindly given by Dr Jean Deutsch). The histochemical (X-gal) and immunolocalization experiments (which were based on the use of a monoclonal antibody directed against β-galactosidase) gave a similar pattern of staining. Fat bodies were dissected in PBS and fixed in 4% paraformaldehyde, 2 mM $MgSO_4$, 1 mM EGTA and 0.1 M PIPES buffer for 15 min. They were

washed three times for 5 min in PBS and permeabilized by a 2 h incubation in PBT A (1% BSA, 0.1% Triton X-100 in PBS). An anti-dorsal monoclonal antibody was applied to the fat bodies at a 1:50 dilution in PBT A and incubated overnight at 4°C. The preparation was then washed three times for 30 min in PBT B (0.1% BSA, 0.1% Triton X-100 in PBS) with 2% sheep serum. The second antibody was an alkaline phosphatase-linked sheep anti-mouse IgG (Boehringer Mannheim, Germany). It was first pre-absorbed on fixed fat body and then diluted 1:500 and applied for 4 h to fat body in PBT B at room temperature. The preparation was fixed for 10 min in 0.5% glutaraldehyde in PBS, washed three times in AP-Sol (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Triton X-100) and incubated for 2 h in the staining solution (0.34 mg/ml NBT, 0.17 mg/ml X-phosphate in AP-Sol). The fat bodies were subsequently mounted in glycerol.

Hemocyte preparation

Third instar wandering larvae were punctured and a small drop of hemolymph was deposited on a glass slide. The preparation was covered immediately by a fragment of coverslip and observed in phase-contrast microscopy.

RNA preparation and analysis

Crosses were performed at 25°C; wandering third instar larvae or 24–48 h adult flies of the appropriate genotype were collected. They were stored at −80°C until extraction. Total RNA was prepared from five to ten flies using a LiCl–urea-based protocol (Richards *et al.*, 1983). Poly(A)-enriched RNA was isolated and analyzed as described (Reichhart *et al.*, 1989). The RNA samples were fractionated by denaturing electrophoresis in 1% agarose–formaldehyde gels with MOPS buffer (Corces *et al.*, 1981) and transferred to nylon membranes (Positive membrane, Appligene). The filters were hybridized overnight at 42°C with either a nick-translated cDNA probe in 50% formamide, 6× SSC, 5× Denhardt's, 100 μg/ml denatured salmon sperm DNA, 0.1% SDS, or an end-labeled oligonucleotide probe in the same solution without formamide. They were subsequently washed three times for 15 min at 42°C in 2× SSC, 0.1% SDS, and then for 20 min at 70°C in 0.2× SSC, 0.1% SDS in the case of a cDNA probe, and in respectively the same solutions three times at room temperature and for 20 min at 42°C for oligonucleotide probes. The following probes were used: a diptericin cDNA (Wicker *et al.*, 1990), a 654 bp *dl* cDNA corresponding to amino acids 61–279, an rp49 cDNA (a PCR fragment of ~400 bp generated between two oligonucleotides designed after the rp49 coding sequence; O'Connell and Rosbash, 1984) and a 21mer oligonucleotide (5′-GATT-CCCAGTCCCTGGATTGT-3′) complementary to part of the coding sequence of cecropins A1 and A2 (see Kylsten *et al.*, 1990).

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References

Anderson,K.V. and Nüsslein-Volhard,C. (1984) *Nature*, **311**, 223–227.

Anderson,K.V. and Nüsslein-Volhard,C. (1986) In Gall,J. (ed.), *Gametogenesis and the Early Embryo*. Alan R.Liss, New York, pp. 177–194.

Baeuerle,P.A. and Baltimore,D. (1991) In Cohen,P. and Foulkes,J.G. (eds), *The Hormonal Control Regulation of Gene Transcription*. Elsevier Science Publishers, Amsterdam, pp. 423–446.

Chasan,R., Jin,Y. and Anderson,K.V. (1992) *Development*, **115**, 607–616.

Cociancich,S., Bulet,P., Hetru,C. and Hoffmann,J.A. (1994) *Parasitol. Today*, **10**, 132–139.

Cogswell,P.C., Scheiman,R.I. and Baldwin,A.S.,Jr (1993) *J. Immunol.*, **150**, 2794.

Corces,V., Pellicer,A., Axel,R. and Meselson,M. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 7038–7042.

Dimarcq,J.L., Hoffmann,D., Meister,M., Bulet,P., Lanot,R., Reichhart,J.M. and Hoffmann,J.A. (1994) *Eur. J. Biochem.*, **221**, 201–209.

Engström,Y., Kadalayil,L., Sun,S.C., Samakovlis,C., Hultmark,D. and Faye,I. (1993) *J. Mol. Biol.*, **232**, 327–333.

Ferguson,E.L. and Anderson,K.V. (1991) *Curr. Top. Dev. Biol.*, **25**, 17–43.

Garzino,V., Pereira,A., Laurenti,P., Graba,Y., Levis,R.W., Le Parco,Y. and Pradel,J. (1992) *EMBO J.*, **11**, 4471–4479.

Gateff,E. (1978) *Science*, **200**, 1448–1459.

Gay,N.J. and Keith,F.J. (1991) *Nature*, **351**, 355–356.

Geisler,R., Bergmann,A., Hiromi,Y. and Nüsslein-Volhard,C. (1992) *Cell*, **71**, 613–621.

Georgel,P., Meister,M., Kappler,C., Lemaitre,B., Reichhart,J.M. and Hoffmann,J.A. (1993) *Biochem. Biophys. Res. Commun.*, **197**, 508–517.

Gertulla,S., Jin,Y. and Anderson,K.V. (1988) *Genetics*, **119**, 123–133.

Gilmore,T. (1994) In Goodbourn,S. (ed.), *Frontiers in Molecular Biology: Eukaryotic Gene Transcription*. Oxford University Press, Oxford, UK, in press.

Gosh,S. and Baltimore,D. (1990) *Nature*, **344**, 678–682.

Govind,S. and Steward,R. (1991) *Trends Genet.*, **7**, 119–125.

Govind,S., Whalen,A.M. and Steward,R. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 7861–7865.

Grilli,M., Chiu,J.J.S. and Lenardo,M.J. (1993) *Int. Rev. Cytol.*, **143**, 1–62.

Hannink,M. and Temin,H.M. (1990) *Oncogene*, **5**, 1843–1850.

Hashimoto,C., Hudson,K.L. and Anderson,K.V. (1988) *Cell*, **52**, 269–279.

Hashimoto,C., Gerttula,S. and Anderson,K.V. (1991) *Development*, **111**, 1021–1028.

Hecht,P.M. and Anderson,K.V. (1993) *Genetics*, **135**, 405–417.

Hiromi,Y., Kuroiwa,A. and Gehring,W.J. (1985) *Cell*, **43**, 603–613.

Hoffmann,J.A., Hetru,C. and Reichhart,J.M. (1993) *FEBS Lett.*, **325**, 63–66.

Hultmark,D. (1993) *Trends Genet.*, **9**, 178–183.

Ip,T.Y., Reach,M., Engström,Y., Kadalayil,L., Cai,H., Gonzalez-Crespo,S., Tatei,K. and Levine,M. (1993) *Cell*, **75**, 753–763.

Isoda,K., Roth,S. and Nüsslein-Volhard,C. (1992) *Genes Dev.*, **6**, 619–630.

Isshiki,H., Akira,S., Sugita,T., Nishio,Y., Hashimoto,S., Pawlowski,T., Suematsu,S. and Kishimoto,T. (1991) *New Biol.*, **3**, 63–70.

Jiang,J. and Levine,M. (1993) *Cell*, **72**, 741–752.

Kappler,C., Meister,M., Lagueux,M., Gateff,E., Hoffmann,J.A. and Reichhart,M. (1993) *EMBO J.*, **12**, 1561–1568.

Kerr,L.D., Inoue,J.-I., David,N., Link,E., Baeuerle,P.A., Bose,H.R.,Jr and Verma,I.M. (1991) *Genes Dev.*, **5**, 1464–1476.

Kidd,S. (1992) *Cell*, **71**, 623–635.

Kylsten,P., Samakovlis,C. and Hultmark,D. (1990) *EMBO J.*, **9**, 217–224.

Letsou,A., Alexander,S., Orth,K. and Wasserman,S.A. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 810–814.

Lindsley,D.L. and Zimm,G.G. (1992) *The Genome of Drosophila melanogaster*. Academic Press, San Diego, CA.

MacDonald,N.J. *et al.* (1990) *Cell*, **60**, 767–779.

Morisato,D. and Anderson,K.V. (1994) *Cell*, **76**, 677–688.

Nappi,A.J. and Carton,Y. (1986) In Brehélin,M. (ed.), *Immunity in Invertebrates*. Springer Verlag, Heidelberg, pp. 171–187.

Nüsslein-Volhard,C. (1979) In Koenigsberg,I. and Subtelney,S. (eds), *Determinants of Spatial Organization*. Academic Press, New York, pp. 185–211.

O'Connell,P. and Rosbash,M. (1984) *Nucleic Acids Res.*, **12**, 5495–5513.

Reichhart,J.M., Essrich,M., Dimarcq,J.L., Hoffmann,D., Hoffmann,J.A. and Lagueux,M. (1989) *Eur. J. Biochem.*, **182**, 423–427.

Reichhart,J.M., Meister,M., Dimarcq,J.L., Zachary,D., Hoffmann,D., Ruiz,C., Richards,G. and Hoffmann,J.A. (1992) *EMBO J.*, **11**, 1468–1477.

Reichhart,J.M., Georgel,P., Meister,M., Lemaitre,B., Kappler,C. and Hoffmann,J.A. (1993) *C.R. Acad. Sci. Paris*, **316**, 1207–1217.

Richards,G., Cassab,A., Bourouis,M., Jarry,B. and Dissous,C. (1983) *EMBO J.*, **2**, 2137–2142.

Rizki,T.M. (1957) *J. Morphol.*, **100**, 459–472.

Rizki,T.M. and Rizki,R.M. (1978) In Ashburner,M. and Wright,T.R.F. (eds), *The Genetics and Biology of Drosophila*. Academic Press, New York, Vol. 2b, pp. 277–313.

Rizki,T.M. and Rizki,R.M. (1992) *Dev. Comp. Immunol.*, **16**, 103–110.

Rizki,T.M., Rizki,R.M. and Grell,E.H. (1980) *Roux Arch. Dev. Biol.*, **188**, 91–99.

Roth,S., Stein,D. and Nüsslein-Volhard,C. (1989) *Cell*, **59**, 1189–1202.

Roth,S., Hiromi,Y., Godt,D. and Nüsslein-Volhard,C. (1991) *Development*, **112**, 371–388.

Rubin,G.M. and Spradling,A.C. (1983) *Nucleic Acids Res.*, **11**, 6341–6351.

Ruohola-Baker,H., Jan,L.Y. and Jan,Y.N. (1994) *Trends Genet.*, **10**, 89–94.
Rushlow,C.A., Han,K., Manley,L. and Levine,M. (1989) *Cell*, **59**, 1165–1177.
Schmitz,M.L., Henkel,T. and Baeuerle,P.A. (1991) *Trends Cell Biol.*, **1**, 130–137.
Schneider,D.S., Hudson,K.L., Lin,T.Y. and Anderson,K.V. (1991) *Genes Dev.*, **5**, 797–807.
Schneider,D.S., Jin,Y., Morisato,D. and Anderson,K.V. (1994) *Development*, **120**, 1243–1250.
Schüpbach,T. and Wieschaus,E. (1989) *Genetics*, **121**, 101–117.
Shelton,C.A. and Wasserman,S.A. (1993) *Cell*, **72**, 515–525.
Shirakawa,F.U. and Mizel,S.B. (1989) *Mol. Cell. Biol.*, **9**, 2424–2430.
Smith,C.L. and DeLotto,R. (1994) *Nature*, **368**, 548–551.
Sparrow,J.C. (1978) In Ashburner,M. and Wright,T.R.F (eds), *The Genetics and Biology of Drosophila*. Academic Press, New York, Vol. 2b, pp. 277–313.
Stein,D. and Nüsslein-Volhard,C. (1992) *Cell*, **68**, 429–440.
Stein,D., Roth,S., Vogelsang,E. and Nüsslein-Volhard,C. (1991) *Cell*, **65**, 725–735.
Steward,R. (1987) *Science*, **238**, 692–694.
Steward,R. (1989) *Cell*, **59**, 1179–1188.
Steward,R. and Govind,S. (1993) *Curr. Opin. Genet. Dev.*, **3**, 556–561.
Steward,R., McNally,F. and Schedl,P. (1984) *Nature*, **311**, 262–265.
Sugumaran,M. and Kanost,M.R. (1993) In Beckage,N.E., Thompson,S.N. and Federici,B.A. (eds), *Parasites and Pathogens of Insects*. Academic Press, New York, Vol. 1, pp. 317–342.
Sun,S.C., Lindström,I., Lee,J.Y. and Faye,I. (1991) *Eur. J. Biochem.*, **196**, 247–254.
Szabad,J., Erdelyi,M., Hoffmann,G., Szidonya,J.A. and Wright,T.R.F. (1989) *Genetics*, **122**, 823–835.
Ten,R.M., Paya,C.V., Israel,N., Le Bail,O., Mattei,M.G., Virelizier,J.L., Kourilsky,P. and Israel,A. (1992) *EMBO J.*, **11**, 195–203.
Tryselius,Y., Samakovlis,C., Kimbrell,D.A. and Hultmark,D. (1992) *Eur. J. Biochem.*, **204**, 395–399.
Wasserman,S.A. (1993) *Mol. Biol. Cell.*, **4**, 767–771.
Watson,K.L., Johnson,T.K. and Denell,R.E. (1991) *Dev. Genet.*, **12**, 173–187.
Whalen,A.M. and Steward,R. (1993) *J. Cell Biol.*, **123**, 523–534.
Whitten,J.M. (1964) *J. Insect Physiol.*, **10**, 447–469.
Wicker,C., Reichhart,J.M., Hoffmann,D., Hultmark,D., Samakovlis,C. and Hoffmann,J.A. (1990) *J. Biol. Chem.*, **265**, 22493–22498.
Wright,T.R.F., Hodgetts,R.B. and Sherald,A.F. (1976) *Genetics*, **84**, 267–285.

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