Cell, Vol. 86, 973–983, September 20, 1996, Copyright ©1996 by Cell Press

The Dorsoventral Regulatory Gene Cassette *spätzle/Toll/cactus* Controls the Potent Antifungal Response in Drosophila Adults

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Summary

The cytokine-induced activation cascade of NF-κB in mammals and the activation of the morphogen dorsal in Drosophila embryos show striking structural and functional similarities (Toll/IL-1, Cactus/I-κB, and dorsal/NF-κB). Here we demonstrate that these parallels extend to the immune response of Drosophila. In particular, the intracellular components of the dorsoventral signaling pathway (except for dorsal) and the extracellular Toll ligand, spätzle, control expression of the antifungal peptide gene drosomycin in adults. We also show that mutations in the Toll signaling pathway dramatically reduce survival after fungal infection. Antibacterial genes are induced either by a distinct pathway involving the *immune deficiency* gene (*imd*) or by combined activation of both imd and dorsoventral pathways.

Introduction

Insects have developed an efficient host defense against microorganisms that relies on three major responses: proteolytic cascades, namely the coagulation and the phenoloxidase cascades, which are immediately induced upon injury; phagocytosis and encapsulation of invading microorganisms by circulating blood cells; and the rapid and transient synthesis, following injury, of a battery of potent antimicrobial peptides (reviewed by Hultmark, 1993; Boman, 1995; Hoffmann, 1995). These molecules are primarily produced by the fat body, a functional analog of the mammalian liver, and are secreted into the blood. Recent studies have identified in Drosophila two functionally distinct classes of inducible antimicrobial molecules: antibacterial peptides, namely cecropins (Kylsten et al., 1990; Tryselius et al., 1992), diptericin (Wicker et al., 1990), drosocin (Bulet et al., 1993), attacin (Asling et al., 1995), insect defensin (Dimarcq et al., 1994), and an antifungal peptide, drosomycin (Fehlbaum et al., 1994).

The elucidation of the control mechanisms that lead to the rapid synthesis of the antimicrobial peptides after septic injury is one of the challenges in this field. We have recently described a recessive mutation, *immune deficiency* (*imd*), which impairs the inducibility of all genes encoding antibacterial peptides during the immune response of Drosophila. This gene has not yet been cloned but, interestingly, the antifungal peptide drosomycin remains fully inducible in homozygous *imd* mutants, a result which points to the existence of different pathways leading to expression of the antifungal and antibacterial peptide genes (Lemaitre et al., 1995a).

We have undertaken the characterization of these pathways. For this, we were guided by the parallels that exist between the cytokine-induced activation cascade of NF-κB during the inflammatory response in mammals (reviewed by Siebenlist et al., 1994; Baeuerle and Henkel, 1994) and the activation of the morphogen dorsal during embryonic dorsoventral patterning in Drosophila (reviewed by Wasserman, 1993; Morisato and Anderson, 1995). These parallels can be summarized as follows (see references above): first, NF-κB and dorsal (dl; Steward, 1987) are members of the Rel family of rapidly inducible transactivators; second, NF-κB and dl are retained in the cytoplasm by the inhibitory proteins I-κB and cactus, respectively (cact; Kidd, 1992; Geisler et al., 1992), which are structurally related; third, once NF-κB and dorsal are released from I-κB or cactus, they translocate into the nucleus and interact with target promoters via closely related κB-binding sites; fourth, dissociation of the NF-κB–I-κB and dl–cact complexes is induced in a signal-dependent manner via transmembrane receptors: significantly, two of the well-established receptors, the interleukin-1 (IL-1) receptor which mediates nuclear transport of NF-κB in mammalian lymphocytes and the Toll receptor (Tl; Hashimoto et al., 1988) which mediates nuclear import of dl, share sequence homology in their cytoplasmic domains (Gay and Keith, 1991; Heguy et al., 1992); and, fifth, signaling through these two receptors may involve protein kinases such as interleukin-receptor associated kinase (IRAK; Cao et al., 1996) and the *pelle* gene product (pll; Shelton and Wasserman, 1993), which also share sequence similarities. The idea that these parallels could be extended to the immune response of Drosophila became attractive when κB-binding sites were observed in the promoters of the genes encoding antibacterial peptides (Sun et al., 1991; reviewed by Hultmark, 1993). These sites were shown to play a pivotal role in the bacterial induction of the diptericin and the cecropin genes (Kappler et al., 1993; Engström et al., 1993). In addition, the immune responsive fat body cells of Drosophila were found to express the two genes encoding Rel proteins: dorsal, initially identified as the dorsoventral morphogen (Reichhart et al., 1993), and Dif (for dorsal-related immunity factor; Ip et al., 1993). Following an immune challenge, both proteins undergo a rapid translocation from the cytoplasm to the nucleus in these cells, and the *dl* and *dif* genes are induced (Ip et al., 1993; Reichhart et al., 1993; Petersen et al., 1995). Interestingly, in *Tl* gain-of-function mutants in which the dl pathway is activated in a signal-independent fashion, both proteins are constitutively present in the nucleus of the fat body cells (Ip et al., 1993; Lemaitre et al., 1995b). Intriguingly though, neither the diptericin nor the cecropin gene is constitutively expressed in these mutants (Lemaitre et al., 1995b).

In the present study, we have analyzed the expression of both the antifungal and antibacterial peptide genes

in strains carrying mutations that affect dorsoventral patterning in the embryo. We show that the embryonic regulatory pathway comprising the gene products between the Tl ligand spätzle (spz; Morisato and Anderson, 1994; Schneider et al., 1994) and cact, but not the genes acting upstream or downstream, play a major role in the control of the antifungal peptide gene drosomycin. Our results confirm the existence of basic differences in the control of expression of the antifungal versus the antibacterial peptides. They also point to unexpected discrepancies in the control of expression of some of the antibacterial genes in adults: while the expression of all these genes was dependent on the product of the *imd* gene (Lemaitre et al., 1995a), the full induction of cecropin A genes, but not that of diptericin, additionally required components of the dorsoventral pathway. We show furthermore that flies mutant for these components have a dramatically reduced resistance to fungal, but not to bacterial, infection. Flies mutant for both the imd and the Tl signaling pathways fail to express to a significant extent any of the antimicrobial genes and rapidly succumb to either fungal or bacterial infections, indicating that these pathways are essential for antimicrobial resistance in Drosophila. Consistent with our inference that the dorsoventral regulatory gene cassette extending from *spz* to *cact* is involved in the antimicrobial response of Drosophila adults, we show that these genes are all expressed in adults and that their expression is up-regulated by immune challenge.

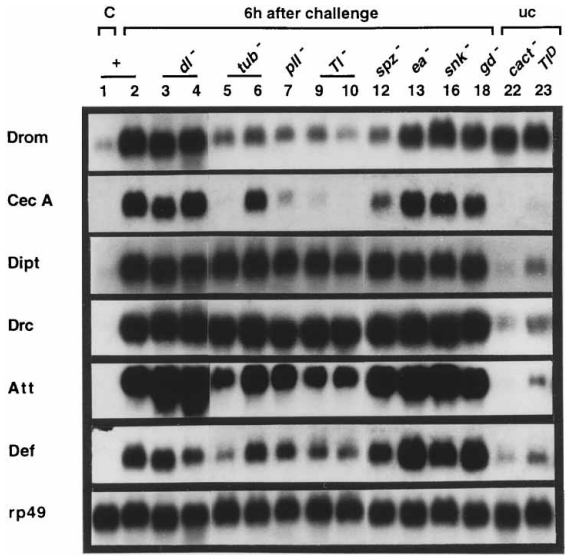

Figure 1. Transcriptional Profiles of Antimicrobial Genes in Wild-Type and Dorsoventral Mutant Adults

A representative Northern blot of total RNA extracted from bacteria-challenged wild-type and mutant adults. The blot was successively hybridized with the following nick-translated cDNA probes: Drom, drosomycin; Cec A, cecropin A1; Dipt, diptericin; Drc, drosocin; Att, attacin; Def, defensin; rp49. C, control; uc, unchallenged. Lanes are as follows: 1 and 2, Oregon^R (Or^R); 3, dl^1/dl^1; 4, $dl^T/Df(dl)$; 5, tub^{238}/tub^{118}; 6, tub^3/tub^{118}; 7, pll^{078}/pll^{21}; 9, $Tl^{r632}/Tl^{1\text{-}RXA}$ (29°C); 10, Tl^{r444}/Tl^{9QURE} (29°C); 12, spz^{rm7}/spz^{rm7}; 13, ea^1/ea^1; 16, snk^{073}/snk^{073}; 18, gd^7/gd^7; 22, $cact^{A2}/cact^{A2}$; 23, $Tl^{10b}/+$.

Results

The results reported in this study were obtained with adult Drosophila. Two types of mutants were analyzed: first, strains carrying strong loss-of-function mutations of *gastrulation defective* (*gd*), *snake* (*snk*; DeLotto and Spierer, 1986), *easter* (*ea*; Chasan and Anderson, 1989), *spz*, *Tl*, *tube* (*tub*; Letsou et al., 1991), *pll*, and *dl*, which are known to block the dorsoventral signaling pathway, leading to completely dorsalized embryos; second, gain-of-function mutations in *Tl* (Tl^D) and loss-of-function mutations in *cact* that are strongly ventralizing (see references in Experimental Procedures). Dorsalizing mutants were challenged by an injection of bacteria, which induces both the antifungal and the antibacterial genes (Fehlbaum et al., 1994; Lemaitre et al., 1995a), whereas ventralizing mutants were analyzed in the absence of challenge. We have examined antimicrobial gene expression by Northern blot analysis and followed the survival rate in dorsoventral and *imd* mutants infected with either bacteria or fungi. We have also studied the expression of the dorsoventral signaling genes after bacterial challenge.

Components of the Dorsoventral Pathway Control the drosomycin Gene

A first striking result was obtained when drosomycin expression was analyzed in Tl^D (Tl^{10b} and Tl^{9Q}) gain-of-function and *cact*-deficient adults, in which the antifungal gene was found to be constitutively expressed in the absence of immune challenge. The level of expression was similar to that induced by bacterial challenge in wild-type adults (Figure 1; for a more detailed analysis, see Figure 2). To ascertain that drosomycin was actually synthesized in Tl^D mutants, we extracted the peptide from these insects, purified it by high pressure liquid chromatography, and quantified it by optical density measurements with reference to standard drosomycin (Fehlbaum et al., 1994). The following results were obtained for 400 adult males: unchallenged wild-type adults, no detectable drosomycin; bacteria-challenged wild-type adults, 7.6 nmol; unchallenged Tl^{10b} mutants, 5 nmol. These results corroborate the data obtained by RNA measurements on the constitutive expression of the drosomycin gene in Tl^{10b} mutants and demonstrate that in these experiments the peptide was produced when the gene was transcribed.

When *spz*-, *Tl*-, *tub*-, and *pll*-deficient mutant adults were bacteria challenged, the level of induction of the drosomycin gene was significantly lower (4- to 5-fold) than in wild-type insects (Figures 1 and 2). In agreement with these results, bacteria-challenged *Tl* adults contained only 0.9 nmol of peptide per 400 insects (i.e., 12% of the value for challenged wild type). We do not attribute the lower level of induction to the general genetic backgrounds of these mutants, since two different alleles were tested for each gene (Figure 2) and flies carrying only the genetic markers showed a wild-type level of induction for the drosomycin gene after challenge (data not shown; see Experimental Procedures for the list of markers). As reported for embryonic development, one of the *Tl* alleles that we tested (Tl^{r632}) was also temperature sensitive for the expression of the drosomycin gene. The immune-induced expression of this

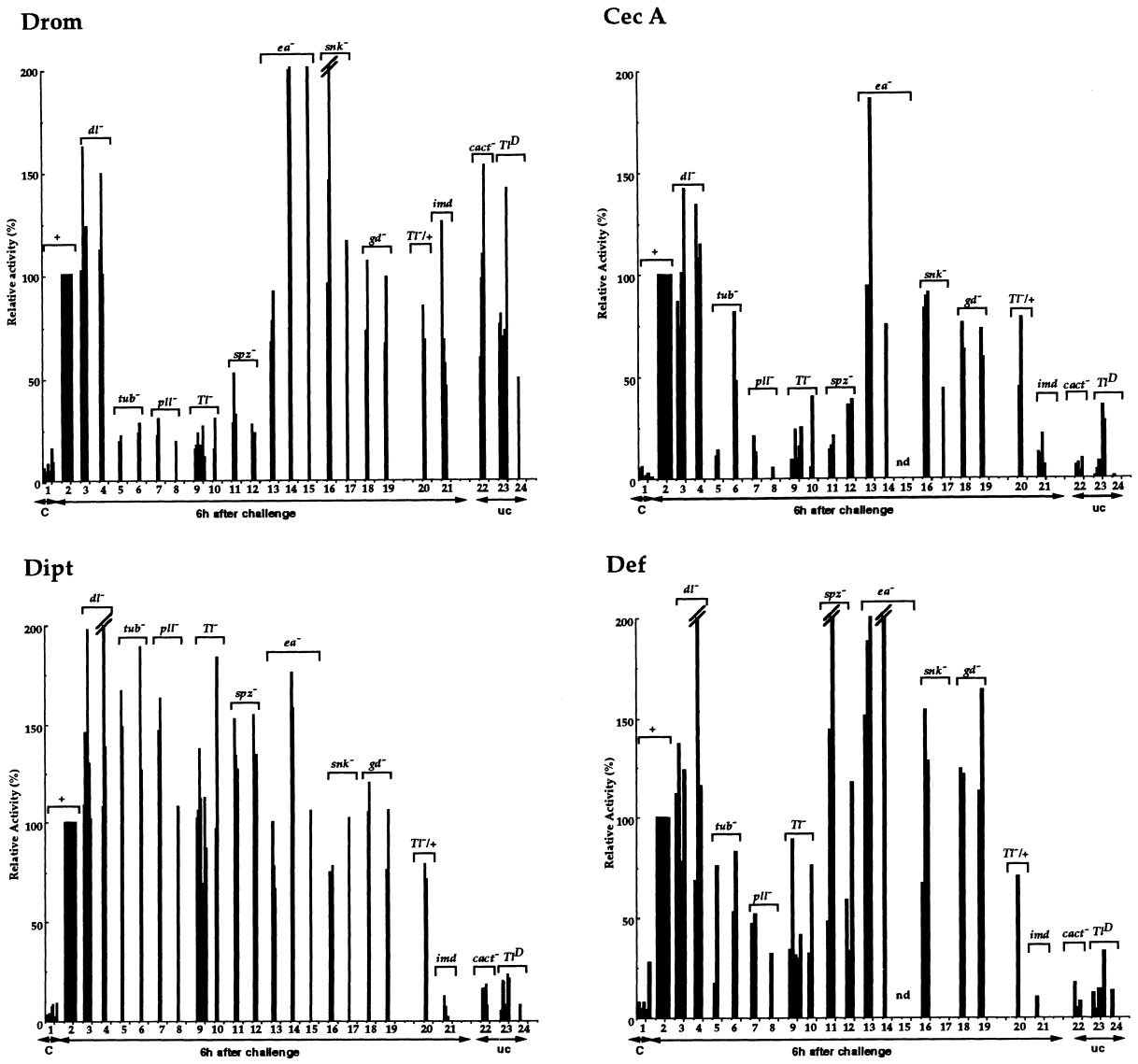

Figure 2. Expression of Antimicrobial Genes in Wild-Type and Dorsoventral Mutant Adults

The signals on several Northern blots similar to that presented in Figure 1 were quantified by a Bio-Imager system. In each experiment, the signals of the immune gene expression were normalized with the corresponding value of the rp49 signal. The levels of expression in 6 hr bacteria-challenged wild-type adults were standardized as 100 and the results are given as relative activity (percent). Each bar corresponds to an independent experiment. Analyses of Northern blots for drosomycin (Drom), cecropin A1 (Cec A), diptericin (Dipt), and defensin (Def) gene expression are presented. Results obtained for drosocin and attacin were similar to those for diptericin and defensin, respectively (data not shown). C, control; uc, unchallenged. Lanes are as follows: 1 and 2, Or^R; 3, dl^1/dl^1; 4, $dl^7/Df(dl)$; 5, tub^{238}/tub^{118}; 6, tub^3/tub^{118}; 7, pll^{078}/pll^{21}; 8, pll^{078}/pll^{rm8}; 9, $Tl^{r632}/Tl^{1\text{-}RXA}$ (29°C); 10, Tl^{r444}/Tl^{9QURE} (29°C); 11, spz^{197}/spz^{197}; 12, spz^{rm7}/spz^{rm7}; 13, ea^1/ea^1; 14, ea^{818}/ea^{818} (29°C); 15, ea^7/ea^2; 16, snk^{073}/snk^{073}; 17, snk^{rm4}/snk^{229}; 18, gd^7/gd^7; 19, gd^8/gd^8; 20, $Tl^{1\text{-}RXA}/+$; 21, *imd/imd*; 22, $cact^{A2}/cact^{A2}$; 23, $Tl^{10b}/+$; 24, $Tl^{9Q}/+$.

gene was indeed more strongly affected in *Tl*-deficient mutants when the adults were placed at 29°C, at which temperature the level of inducibility relative to wild type was 15%, as compared with 40% at 18°C. We also noted that *Tl* behaved as a recessive mutant, since heterozygous *Tl*/+ adults exhibited a close to wild-type level of expression of the drosomycin gene (Figure 2).

In contrast with adults deficient in spz, Tl, tub and pll, mutants in the *gd*, *snk* and *ea* genes, which lie upstream in the dorsoventral patterning cascade, show a wild-type response to bacterial challenge (Figures 1 and 2). The *ea* and *snk* genes encode serine proteases (DeLotto and Spierer, 1986; Chasan and Anderson, 1989), that are involved in processing of the *spz* gene product. Since the alleles of *ea* and *snk* used in our study are point mutations in the catalytic site, which result in nonfunctional proteases (Jin and Anderson, 1990; Smith et al., 1994), our results indicate that neither *ea* nor *snk* is required for the control of drosomycin gene expression. Finally, in *dl* mutants, drosomycin gene induction was not affected.

Since the preceding data were based on a single timepoint (6 hr postchallenge), we wondered whether in mutants in the dorsoventral regulatory pathway the time

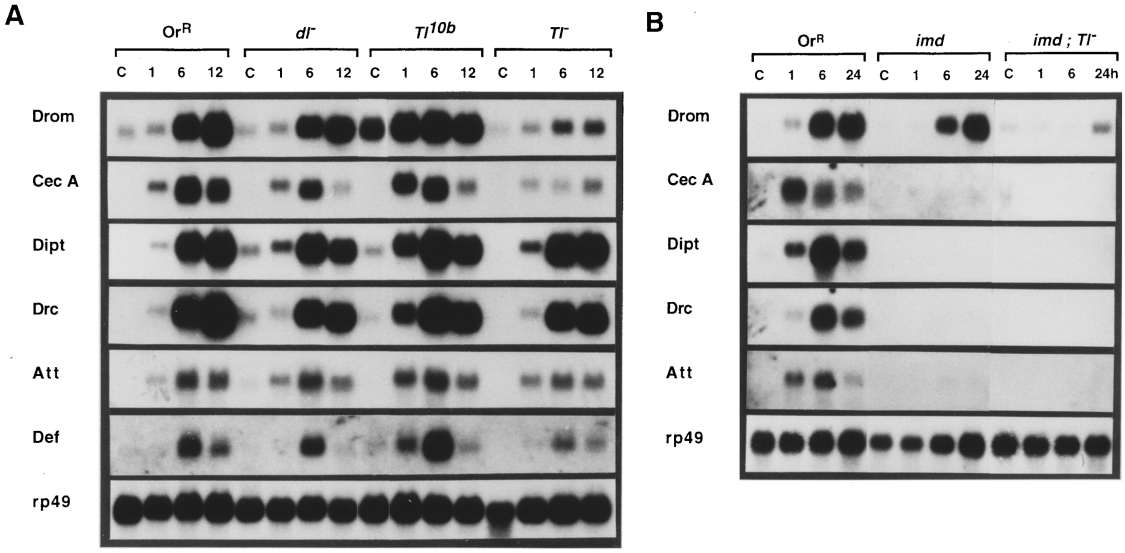

Figure 3. Time Course Analysis of Antimicrobial Gene Expression in Wild-Type and Mutant Adults

Northern blot analysis of total RNA extracted at different time intervals after challenge (as indicated). Flies were bacteria challenged and processed together. (A) and (B) were obtained separately.
(A) Or^R, dl^1/dl^1 (dl^-), $Tl^{10b}/+$ (Tl^{10b}), $Tl^{r632}/Tl^{1\text{-}RXA}$ (Tl^-).
(B) Or^R, *imd/imd* (*imd*) and *imd/imd*; Tl^{632}/Tl^{9QURE} (*imd*; Tl^-).

course of drosomycin might also be affected (Figure 3A). These experiments were restricted to gain-of-function and loss-of-function *Tl* mutants and to *dl* mutants. No striking discrepancies were observed, and the results basically confirm the data presented above in that drosomycin induction is low in a *Tl*-deficient background, similar to that of wild-type in *dl* mutants, and the gene is constitutively expressed in Tl^{10b} mutants. Interestingly, the level of expression was increased beyond the constitutive level after bacterial challenge.

Altogether our results indicate that the embryonic regulatory pathway, comprising the gene products between spz and cact but not the genes acting upstream or downstream, is involved in the induction of the drosomycin gene in adults.

Distinct Pathways Control the Expression of the Antibacterial Genes in Adults

The Northern blots used above were repeatedly dehybridized and successively probed with cecropin A1, diptericin, drosocin, attacin, and defensin cDNAs. We first observed that, in contrast with the drosomycin gene, no strong constitutive expression occurred for any of the antibacterial peptide genes in gain-of-function *Tl* and *cact*-deficient mutant adults (Figures 1 and 2). However, the level of constitutive expression of all antibacterial genes in these mutants was higher than the basal level (roughly 2- to 5-fold higher; Figure 2).

The induction of the cecropin A genes (two cecropin A genes, A1 and A2, differ in their nucleotide sequence but code for the same protein; Kylsten et al., 1990) by bacterial challenge was markedly reduced in adults deficient for *spz*, *Tl*, *tub*, and *pll*, the level of transcripts being approximately 4-fold lower than in wild-type flies. In contrast, cecropin A gene induction was not affected in mutants deficient in *gd*, *snk*, *ea*, or *dl*. Note that for the allelic combination tub^3/tub^{118}, the inducibility of the cecropin A genes was only slightly affected, in contrast with the tub^{238}/tub^{118}background (Figures 1 and 2). At present we have no explanation for this discrepancy. However, the observation that the inducibility of the drosomycin gene is altered in both *tub* backgrounds tested suggests that the expression of cecropin A is less tightly controlled by the dorsoventral pathway than the antifungal peptide gene. Overall, the pattern of response of the cecropin A genes paralleled that of the drosomycin gene, except that cecropin A was not constitutively expressed in *cact*-deficient and Tl^D gain-of-function mutants.

In contrast with the cecropin A genes, the diptericin and the drosocin genes remained fully inducible in all the mutants tested above, including *spz*, *Tl*, *tub*, and *pll*. The induction of the attacin and defensin genes was slightly weaker in *Tl*, *tub*, and *pll* mutants than in wild-type or *ea*, *snk*, and *dl* mutants, which behaved as wild type. Surprisingly, the inducibility of the last two genes was less altered in *spz* mutants than in *pll*-, *tub*-, and *Tl*-deficient flies. In general, the pattern of expression of these genes was intermediate between that of the cecropin A genes and the diptericin–drosocin group.

Experiments analyzing the time course of the induction of antibacterial peptide genes reinforced these results. In *Tl*-deficient adults, the cecropin A and, to a lesser extent, attacin and defensin genes were only minimally inducible (Figure 3A), in contrast with the diptericin and drosocin genes, which remained fully inducible in this context. No marked constitutive expression was observed in Tl^D gain-of-function mutants which, interestingly, exhibited a more rapid induction of all antibacterial genes after bacterial challenge (compare Tl^{10b} and wild-type adults 1 hr after bacterial challenge in Figure 3A). This last result, as well as the previous observation that

in *cact* and Tl^D mutants antibacterial genes are expressed above basal level, indicates that the Tl pathway may slightly influence the expression of all the antibacterial genes (including diptericin and drosocin). As previously reported (Lemaitre et al., 1995b), the induction of the antibacterial peptide genes in the *dl*-deficient adults was roughly similar to that in wild-type adults.

Altogether, these results strengthen the idea that genes encoding antibacterial peptides are regulated in a manner distinct from that of the gene encoding the antifungal peptide drosomycin. They also demonstrate that the antibacterial genes differ in their requirements for the dorsoventral pathway components. Cecropins A are the only genes that clearly require the *spz–cact* regulatory cassette for expression at the adult stage. However, constitutive activation of the *Tl* signaling pathway, as well as the absence of *cact*, are not sufficient to trigger a strong expression of these genes, in contrast with drosomycin.

The Induction of All Antimicrobial Genes Is Impaired in *imd;Tl* Double Mutant Flies

The drosomycin gene is only minimally expressed in *Tl*-deficient mutants, as shown above, and the expression of antibacterial peptide genes is impaired in *imd* mutants (Lemaitre et al., 1995a). In the hope of generating flies with a severely depressed antimicrobial response, we have constructed lines homozygous for both the *imd* and the *Tl* mutations. Figure 3B shows that in these double mutants the inducibility of all antimicrobial genes by bacterial challenge was severely affected. No signal was detectable on Northern blots for any antibacterial gene for up to 24 hr following challenge, and only a faint signal was observed for drosomycin at 24 hr postchallenge.

Mutations That Affect the Synthesis of Antimicrobial Peptides Dramatically Lower the Resistance of Flies to Infection

We analyzed the survival rate of *imd, Tl* and *imd*;*Tl* mutant adults after fungal and bacterial infections. The insects received one of three treatments: pricking with a clean needle under nonsterile conditions (septic injury) to monitor the effect of injury and associated microorganisms from the environment; pricking with a needle dipped into a concentrated solution of spores of the fungus Aspergillus fumigatus; or pricking with a needle dipped into a concentrated solution of Escherichia coli. Northern blot analysis showed that these treatments all induced in wild-type adults the expression of the antibacterial and antifungal genes, the level of induction, however, being noticeably weaker for the first type of treatment (data not shown). The survival rate was followed over a 6 day period at 29° C. At this temperature, the susceptibility to microbial challenge is highest. The temperature sensitivity of infected adults most likely reflects the temperature-dependent growth of the microorganisms within the infected insects, as it affects all experimental flies regardless of their respective genotypes (data not shown).

As illustrated in Figure 4A, a septic injury did not noticeably affect wild-type adults or *imd* and *Tl* mutants. The survival of double mutants was more drastically affected, and only 40% had survived 6 days after septic injury.

Infections with A. fumigatus, a weak pathogen for insects (Vey and Götz, 1986), resulted in about one third mortality in the wild-type population (Figure 4B). Interestingly, the survival of *imd* mutants, in which only the synthesis of antibacterial peptides is impaired, was similar to that of wild type. In sharp contrast, *Tl*-deficient insects were dramatically affected by the fungus. All flies died after 2–3 days and death was clearly associated with uncontrolled fungal development, since flies were covered with fungal hyphae after their death, as illustrated in Figure 5.

The survival rates of wild-type flies and *Tl* mutants, which both produce antibacterial peptides, were not markedly affected after infection with E. coli (Figure 4C). Interestingly, bacterial counts performed on fly extracts over a 24 hr period following infection showed that no bacterial growth had occurred in these insects. The results were strikingly different in experiments with *imd* or *imd*;*Tl* mutants, in which only a few individuals survived after 3 days. In these mutants, bacterial growth was intense, and the number of bacteria was 1000 times higher than in wild-type or *Tl* flies at 24 hr postinfection (Figure 6).

We have extended the studies on the survival rates to the other members of the dorsoventral signaling pathway. Tl^D and *cact* adults, in which the antifungal gene is constitutively expressed (see above), were not studied since they exhibit a lower viability even in the absence of challenge. Table 1 presents the percentage of survival 3 days after fungal or bacterial infection in flies mutated for each component of the pathway. Day 3 was chosen because the difference in survival rates in the preceding experiments (Figure 4) were more marked at this timepoint. Our results show that flies deficient for pll, tub, and spz have a dramatically increased susceptibility to fungal infection, as was observed for *Tl*-deficient mutants. In contrast, in *dl* and *ea* mutants, the survival rate was similar to that of wild-type insects. None of these mutations had a marked effect on the survival rate for bacterial infections (Table 1), which is consistent with our observation that most of the antibacterial peptides remain fully inducible in these mutants (see above). These results underline the correlation between the impairment of drosomycin synthesis and the susceptibility to fungal infection. They also strengthen the inference that the *spz*, *Tl*, *pll*, and *tub*, but not the *ea* or *dl*, gene products are required for the antifungal host defense.

The Dorsoventral Genes Are Induced upon Immune Challenge

It is well established that the transcripts of the dorsoventral genes are present in ovaries and early embryos in relation to their role in dorsoventral patterning during early embryogenesis (reviewed by Morisato and Anderson, 1995). To lend further credit to the conclusion that these genes are involved in the immune response of adult insects, we have verified that they are actually

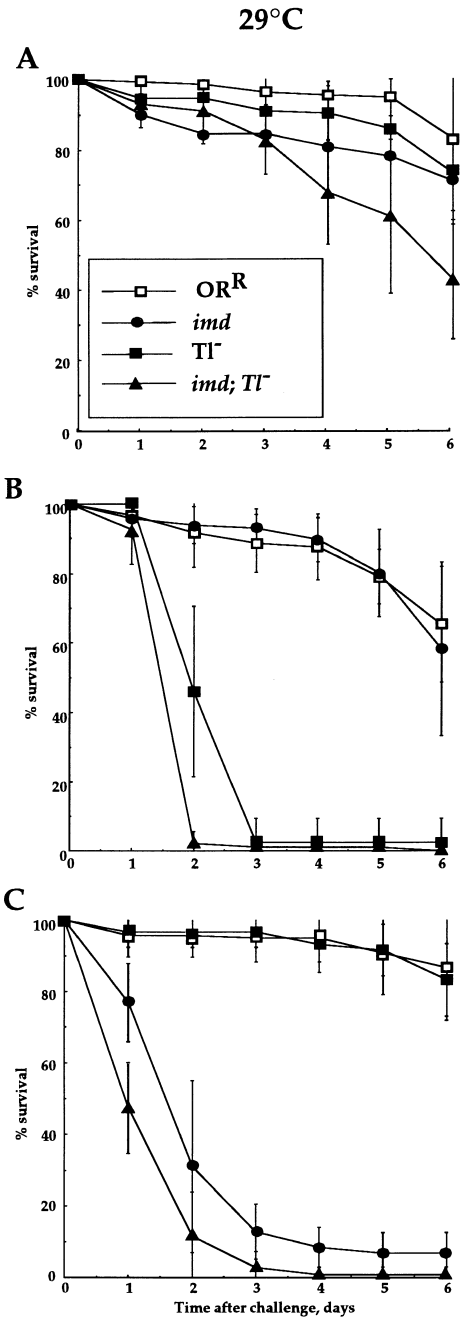

Figure 4. Survival of Wild-Type and Mutant Adults to Bacterial and Fungal Infections

The survival rates of infected *imd/imd* (*imd*); $Tl^{632}/Tl^{1\text{-}RXA}$ (Tl^{-}), *imd/imd*; $Tl^{632}/Tl^{1\text{-}RXA}$ (*imd*; Tl^{-}) mutants and wild-type (Or^{R}) flies are presented with their confidence interval ($p < 0.05$). Groups of 20 adults, aged 2–4 days, were pricked and transferred to a fresh vial every 4 days. The survival rates of untreated mutants were identical to those observed with wild type (>95% after 6 days; data not shown). At least five replicates were used for the determination of the survival rates. Adults were pricked with a needle previously dipped into either water (A), a concentrated solution of spores of A. fumigatus (10^{9} spores per milliliter) (B), or a concentrated solution of E. coli (C).

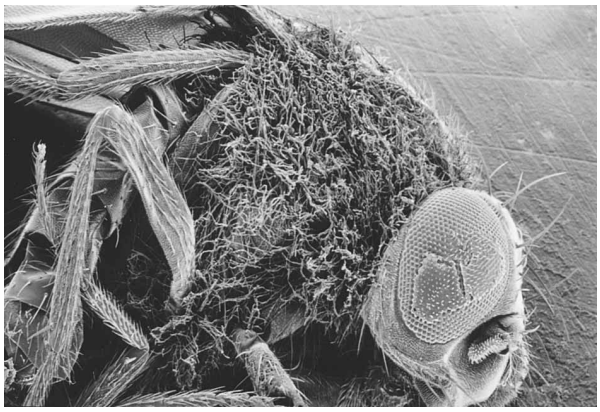

Figure 5. Germinating Hyphes of A. fumigatus on a Dead Drosophila

Scanning electron micrograph of a Drosophila adult that succumbed to infection by A. fumigatus and is covered with germinating hyphae (200× magnification).

expressed at this stage. We have successively probed in Northern blotting experiments poly(A) RNA prepared from control and immune-challenged adult males (to avoid interference with ovarian transcripts) with cDNAs of various genes of the dorsoventral cascade, plus cDNA encoding the Rel protein Dif. Interestingly, we observed that the *cact*, *pll*, *tub*, *Tl*, and *spz* genes are expressed

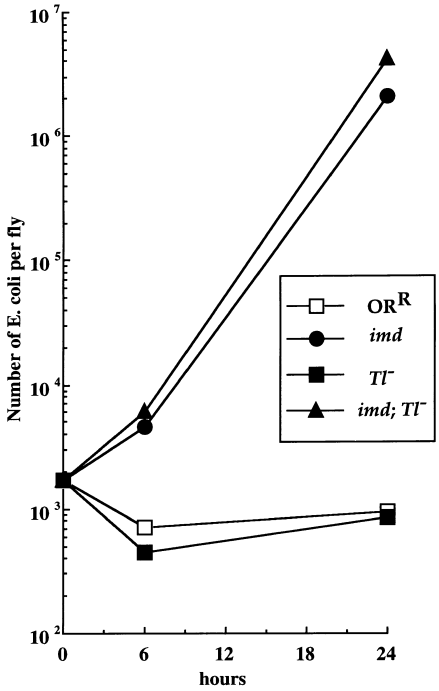

Figure 6. Bacterial Growth in Challenged Wild-Type and Mutant Adults Infected with E. coli

imd/imd, $Tl^{632}/Tl^{1\text{-}RXA}$, and *imd/imd*; Tl^{r632}/Tl^{9QURE} mutants and wild-type (Or^{R}) flies were pricked with a needle dipped into a concentrated solution of E. coli carrying an ampicillin-resistant plasmid. Bacterial counts were obtained by plating an appropriate dilution of homogenates of five adults in phosphate-buffered saline on ampicillin medium. This experiment was repeated several times and yielded similar results.

Table 1. Survival of Dorsoventral Mutant Adults to Bacterial and Fungal Infections

Genotype Tested	Fungal Infection	Bacterial Infection
Or^R	89 (4.2;9)	95 (5.3; 14)
dl^1/dl^T	94 (4.3; 5)	92 (3.0; 6)
pll^{078}/pll^{21}	4 (7.4; 5)	87 (8.5; 8)
tub^{238}/tub^3	3 (5.3; 6)	71 (27; 4)
$Tl^{r632}/Tl^{l\text{-}RXA}$	8 (10.8; 8)	93 (6.6; 9)
spz^{rm7}/spz^{197}	3 (5.6; 7)	84 (11; 9)
ea^1/ea^2	98 (8.8; 5)	87 (5.7; 8)
imd/imd	93 (5.6; 5)	8 (7.4; 13)
imd/imd; $Tl^{632}/Tl^{l\text{-}RXA}$	1 (2.3; 5)	3 (4.4; 6)

The survival rates are given in percentage, with standard deviation and the numbers of measurements in parentheses. The survival rate was measured 3 days after fungal (A. fumigatus) or bacterial (E. coli) infections. The conditions of these experiments were as described in Figure 4.

at a low level at this stage of development (Figure 7). More significantly in the present context, we noted that the transcription of all these genes (except for *tub*) was clearly up-regulated in response to immune challenge.

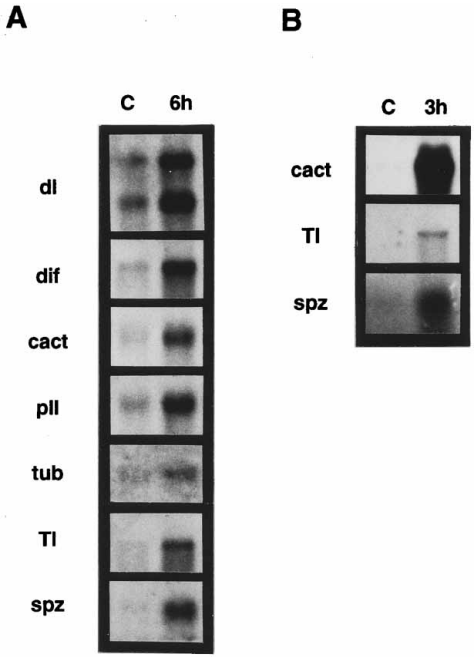

Figure 7. Expression of Dorsoventral Genes in Control and Challenged Adults

Aliquots containing 5 μg of poly(A) RNA from uninduced (0 hr) or bacteria-challenged male adults were fractionated on an agarose–formaldehyde gel and subsequently hybridized with cDNA from the dorsoventral genes and from rp49 as a control. (A) and (B) were obtained from distinct experiments. Equivalent levels of rp49 signals were obtained for each experiment (data not shown). The sizes of the transcripts were similar to published values: *Tl*, 5.3 kb (Hashimoto et al., 1988); *dif*, 2.8 kb (Ip et al., 1993); *dl*, 2.6 and 4.6 kb (Steward, 1987; Reichhart et al., 1993); *cact*, 2.2 kb (Geisler et al., 1992; Kidd, 1992); *pll*, 1.9 kb (Shelton and Wasserman, 1993); *tub*, 2 kb (Letsou et al., 1991); *spz*, 2.2 kb (Morisato and Anderson, 1994). The separating capacity of the gel was not sufficient to distinguish the two splicing isoforms of cactus RNA (Geisler et al., 1992; Kidd, 1992).

The overall level of inducibility 6 hr after bacterial challenge was estimated, by radioactive measurements and comparison with the reference rp49 transcript (data not shown), to be approximately 2- to 5-fold. Our results (Figure 7) also confirm previous data suggesting that the *dl* and *dif* genes are expressed at a basal level in male adults and that bacterial challenge increases their level of expression (Reichhart et al., 1993; Petersen et al., 1995). Antimicrobial peptide genes are mainly expressed in the fat body of adults, a thin tissue difficult to excise at this stage. However, a careful preparation of abdominal dorsal carcass of male flies allows extraction of predominantly adult fat body RNA with minor contaminations from epidermal and muscle RNA. As shown in Figure 7B, we observed that *cact*, *Tl*, and *spz* genes are expressed in such preparations and that this expression is markedly up-regulated by bacterial challenge.

Discussion

Identification of a Pathway Mediating an Antifungal Response

Two major conclusions can be drawn from the analysis of the expression of the drosomycin gene in adults. First, in *Tl* gain-of-function and *cact*-deficient mutants, the drosomycin gene is constitutively expressed and the level of expression is similar to that induced by bacterial challenge in wild-type adults. The activation of the Tl signaling pathway is therefore sufficient for the induction of the antifungal gene. We have shown mutations in Drosophila to mimic an immune challenge, leading to the synthesis of an antimicrobial peptide. Second, loss of function in any of the genes extending in the dorsoventral regulatory cascade from *spz* to *pll* results in a markedly impaired induction of the drosomycin gene after bacterial challenge. These data lead us to conclude that the gene cassette comprising the intracellular part of the dorsoventral pathway (with the exception of dl), plus the extracellular component spz, is involved in the control of transcription of the drosomycin gene (see the model in Figure 8). Our inference is further substantiated by the observation that these genes are actually expressed at the adult stage (most probably in the fat body cells, to judge from the experiments with abdominal carcasses) and that their expression (with the exception of *tub*) is markedly up-regulated by immune challenge. The latter result further indicates that the components of the cascade are themselves controlled at the transcriptional level during the immune response.

Mutations in the *snk* and *ea* genes, which are upstream of *spz*, do not affect the inducibility of the drosomycin gene, indicating either that the *snk* and *ea* gene products are not involved in the induction of the drosomycin gene or that in their absence other gene products can fully substitute for their function. *snk* and *ea* encode serine proteases of the trypsin family (Chasan and Anderson, 1989; DeLotto and Spierer, 1986) and may serve to activate the *spz* gene product by proteolytic cleavage, enabling the processed protein to bind to the Tl receptor (Morisato and Anderson, 1994; Schneider et al., 1994).

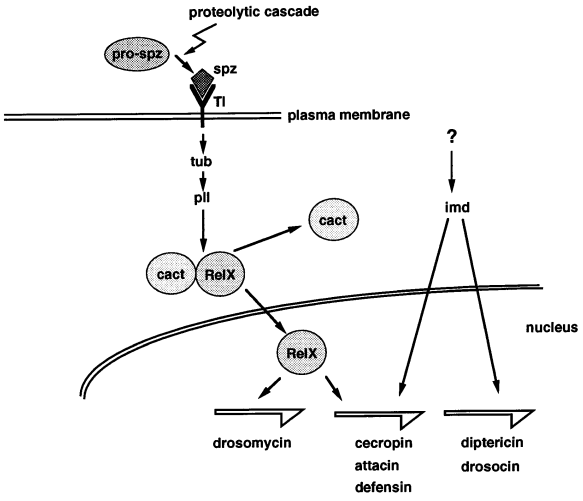

Figure 8. Model for the Control of Expression of Genes Encoding Antimicrobial Peptides in the Drosophila Fat Body of Adults

Two distinct pathways activate the expression of antimicrobial peptide genes in adults. Drosomycin is induced via a Rel protein (possibly Dif or an as yet unidentified protein, but not dl), which is retained in the cytoplasm of the fat body by cact. The dissociation of the cact–RelX complex is mediated by the intracellular *Tl*, *tub*, and *pll* gene products. By analogy with the embryonic system in which spz is present in the perivitelline fluid (reviewed by Morisato and Anderson, 1995), it is proposed that spz is present in the hemolymph, is processed by a protease of a proteolytic cascade induced upon septic injury (e.g., coagulation or phenoloxidase cascades), and acts as a ligand to activate the Tl receptor. The *imd* gene product has not been characterized and its precise role remains an open question. All the genes encoding antibacterial peptides require the *imd* gene product for their induction. In contrast with diptericin and drosocin, the full induction of the cecropin A, defensin, and attacin genes also depends on the Tl pathway. We leave open the question of whether these two regulatory pathways act on the same or distinct transcriptional activator(s).

An attractive hypothesis is that the Drosophila proteolytic cascades initiated by injury, which lead to coagulation or melanization, activate as yet unidentified proteases, different from snk and ea, which cleave the spz protein to its functional form. The observation that serine proteases of the horseshoe crab clotting cascade share sequence similarities with the *ea* serine protease lends some credit to this hypothesis (reviewed by Hecht and Anderson, 1992; Iwanaga, 1993). In the embryonic system, spz is present in the perivitelline space, and we speculate that during the immune response spz is present in the hemolymph and plays a cytokine-like role (Figure 8). The involvement of the *spz* gene in the immune response of adults was unexpected since, in contrast with *cact*, *pll*, *tub*, and *Tl* (Gertulla et al., 1988; Roth et al., 1991; Hecht and Anderson, 1993), the *spz* mutant shows no striking zygotic phenotype.

The partner of *cact* in the drosomycin induction pathway has not yet been identified. The hypothesis that this partner is a member of the Rel family is consistent with the observation that the upstream sequences of the drosomycin gene contain several κB-related binding motifs (L. M., unpublished data). Drosomycin is fully inducible in *dl*-deficient mutants, implying that dl does not play a key role in drosomycin induction. Dif may be a better candidate. Indeed, this Rel protein has also been shown to bind in vitro to the cact protein (Tatei and Levine, 1995). The candidacy of Dif is further supported by the observation that in transfection experiments this protein is a more powerful transactivator for cecropin A1 gene than for diptericin gene expression (Petersen et al., 1995; Gross et al., 1996). This result is in agreement with our present data showing that the induction of cecropin A, but not that of diptericin, requires the dorsoventral gene cascade. To date, no mutants for Dif are available and we have to leave open the question of the nature of the transactivator acting on the drosomycin gene.

A preliminary analysis of the regulation of the antimicrobial peptide genes in larvae indicates that the dorsoventral components are also involved, but point to additional regulatory mechanisms at this stage (unpublished data).

The Tl and imd Signaling Pathways Are Both Required for a Full Antibacterial Response in Adults

The antibacterial response of Drosophila involves the coordinate expression of at least five genes encoding bactericidal peptides. Preliminary data point to the existence of several additional inducible antibacterial peptides in this species (P. Bulet, personal communication). The presence of κB-binding sites in the promoters of all antibacterial genes (reviewed by Hultmark, 1993) and the requirement for the *imd* gene product (Lemaitre et al., 1995a) suggested that a common regulatory mechanism controls the expression of all these genes. The present study points to a more complex picture in adults (see the model in Figure 8). In addition to a functional pathway involving the *imd* gene product, the cecropin A genes, and to a lesser extent the attacin and insect defensin genes, also require the products of the *spz*, *Tl*, *tub*, and *pll* gene cassette, which is clearly dispensable for the induction of the diptericin and the drosocin genes. In the tumorous blood cell line mbn-2, overexpression of Tl^{10b} had been observed to increase transcription of a cecropin A1 reporter gene, which was further stimulated by lipopolysaccharide, a result which also suggests that two cascades exist in the control of this immune gene (Rosetto et al., 1995). We observed that in Tl^{D} gain-of-function and *cact*-deficient mutants, neither the cecropin A nor the attacin and insect defensin genes showed a marked constitutive expression, which indicates that the induction of this group of genes requires the concomitant activation of both pathways (Figure 8). Similarly, data obtained with knockout mice have recently shown that some of the NF-κB-responsive genes are constitutively expressed in mice deficient in the cact homolog, I-κBα, while other genes require additional activation pathways (Beg et al., 1995).

We are aware that the existence of two distinct regulatory pathways controlling the expression of antimicrobial genes raises the question of the possible existence of distinct recognition mechanisms. To date, our information on non-self-recognition in insect immunity is fragmentary, and this area is now becoming a major field of interest (discussed by Hultmark, 1993; Hoffmann, 1995).

Role of the Antimicrobial Peptides in the Resistance to Infection in Drosophila

The presence of antimicrobial peptides has been reported over the last 15 years in as disparate organisms as plants, arthropods, mollusks, amphibians, and mammals. Some of these molecules share sequence similarities bridging the phylogenetic distances (e.g., brevinins, cecropins, and defensins) while others appear specific to a given class of organisms (reviewed by Hetru et al., 1994; Boman et al., 1995; Broekaert et al., 1995). The antimicrobial peptides have been proposed to play a role in limiting bacterial or fungal proliferation and thus in contributing to the host defense. The generation of the *imd*;*Tl* double mutants, in which the synthesis of all antimicrobial peptides is impaired, has provided a model to investigate the in vivo relevance of the immune-induced synthesis of antimicrobial peptides in the host defense. Our results clearly demonstrate that the imd and Tl pathways are both essential for full antimicrobial resistance in Drosophila. Our study also establishes 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. Although we cannot exclude the possibility that these mutations also affect other immune mechanisms (proteolytic cascades, cellular reactions essential for the host defense), our results show that under our experimental conditions the insects could not survive an infection when antimicrobial gene induction was impaired, most likely, as illustrated for E. coli, because they were unable to control the proliferation of the microorganisms.

Concluding Remarks

Several studies have underlined the parallels between the cytokine-induced activation of NF-κB and the Tl-mediated activation of dl. It has also been proposed that these parallels extend to the Drosophila immune response. By providing a demonstration that the components of the dorsoventral pathway play an essential role in the potent antimicrobial response of Drosophila, we confirm that this activation pathway is indeed an ancient regulatory cascade involved in host defense. The recent identification of the N-protein, which plays a role in the resistance of tobacco plants to tobacco mosaic virus, has highlighted sequence similarities between the N-terminal domain of this protein and the cytoplasmic domain of both the IL-1 and Tl receptors (Whitham et al., 1994). By showing that Tl is also involved in the immune reponse, we lend further credit to the idea that the host defense in higher eukaryotes involves a common regulatory pathway, mediated by a homologous protein domain present in these proteins (Dinesh-Kumar et al., 1995).

The powerful genetic system of Drosophila provides an excellent model for further dissection of the control mechanisms of primordial immunity.

Experimental Procedures

Drosophila Stocks

The lines used in this study have been described elsewhere (Anderson and Nüsslein-Volhard, 1984, 1986; Lemaitre et al., 1995a, 1995b). Mutants in *pll*, *tub*, and especially *Tl* have been reported to exhibit some lethality during the larval stage and larvae and pupae of abnormal size (Letsou et al., 1991; Gertulla et al., 1988; Hecht and Anderson, 1993). To obtain *Tl* larvae and adults, we have used two thermosensitive alleles of *Tl* (Tl^{632} and Tl^{r444}) that exhibit a strong phenotype only when raised at 29°C (Gertulla et al., 1988). *Tl*-deficient mutants were reared at 18°C and shifted at the adult stage to 29°C. Since strong alleles of *cact* result in larval/pupal lethality (Roth et al., 1991), we have used here the strongest viable allele of *cact* ($cact^{A2}$). All experiments were performed at 25°C except when otherwise stated. For complete descriptions of the marker genes and balancer chromosomes, see Lindsley and Zimm (1992).

Injury and Survival Experiments

Bacterial challenge was performed by pricking adults with a thin needle previously dipped into a concentrated bacterial culture of E. coli and Micrococcus luteus.

Survival experiments were carried out in the same conditions for each genotype tested. Groups of 20 adults, aged 2–4 days, were challenged and transferred to a fresh vial every 4 days. Flies that died within 3 hr (less than 5% of the total) after challenge were not considered in the analysis. In preliminary experiments, we observed that survival rates could strongly depend on the genetic background. For example, we noted that homozygous *ebony* (*e*) flies exhibited a low viability after challenge as previously reported by Flyg and Boman (1988). To test the survival of the mutation under analysis, we used strains with mutated chromosomes carrying a minimum of markers and exhibiting a good viability.

RNA Preparation and Analysis

Crosses were performed at 25°C, and 2- to 4-day-old adult flies were collected. Total RNA extraction and Northern blotting experiments were performed as in Lemaitre et al. (1995a). The following probes were used: attacin cDNA (Asling et al., 1995), cecropin A1 cDNA (Kylsten et al., 1990), defensin cDNA (Dimarq et al., 1994), diptericin cDNA (Wicker et al., 1990), drosocin cDNA (Bulet et al., 1993), drosomycin cDNA (Fehlbaum et al., 1994), rp49 cDNA (a PCR fragment of approximately 400 bp generated between two oligonucleotides designed after the rp49 coding sequence (O'Connel and Rosbach, 1984). The cecropin A1 probe cross-reacts with cecropin A2 transcripts (Kylsten et al., 1990). We observed that chromosomal markers associated with the mutations that were tested (*b*, *pr, cn*, *bw*, *sp, ru*, *st*, *e*, *ca*, *cu*, *kar*, *red*, *sbd*, *th*, *ri*, *roe*, p^p, *h*, *sr*, *mwh*) did not alter the induction of the genes encoding drosomycin, cecropin A and diptericin. Poly(A) RNA extraction was prepared as in Lemaitre et al. (1995b), except that extraction of total RNA was performed with the RNA Trizol (GIBCO BRL) method.

Acknowledgments

Correspondence should be addressed to J. A. H. The authors are indebted to Dr. Christiane Nüsslein-Volhard, Dr. Kathryn Anderson, and Dr. Steve Wasserman for sending flies carrying dorsoventral mutations or plamids encoding dorsoventral genes, to Dr. Kathy Matthews for sending current stocks of the Bloomington Center, and to Dr. Dan Hultmark and Dr. Mitchel Dushay for the gift of attacin and cecropin A1 cDNAs. We thank our colleagues Pascale Fehlbaum and Dr. Philippe Bulet, who performed the high pressure liquid chromatography purification of the drosomycin peptide, and Dr. René Lanot, Dr. Marie Meister, Dr. Jean Luc Imler, Dr. Sarah Ades, and Dr. Dominique Ferrandon for stimulating discussions. The technical assistance of Reine Klock and Raymonde Syllas is gratefully acknowledged. This work was supported by the Centre National de la Recherche Scientifique, the University Louis Pasteur of Strasbourg, Rhone Poulenc, and the Human Frontiers Science Programme.

Received March 13, 1996; revised August 8, 1996.

References

Anderson, K.V., and Nüsslein-Volhard, C. (1984). Information for the dorso-ventral pattern of the *Drosophila* embryo is stored as maternal mRNA. Nature *311*, 223–227.

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

Asling, B., Dushay, M., and Hultmark, D. (1995). Identification of early genes in the *Drosophila* immune response by PCR-based differential display: the *Attacin A* gene and the evolution of attacin-like proteins. Insect Biochem. Mol. Biol. *25*, 511–518.

Baeuerle, P.A., and Henkel, T. (1994). Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. *12*, 141–179.

Beg, A.A., Sha, W.C., Bronson, S., and Baltimore, D. (1995). Constitutive NF-κB activation, enhanced granulopoiesis, and neonatal lethality in IκBα-deficient mice. Genes Dev. *9*, 2736–2746.

Boman, H.G. (1995). Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. *13*, 61–92.

Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., and Osborn, R.W. (1995). Plant defensins: novel antimicrobial peptides as components of the host defense systeme. Plant Physiol. *108*, 1353–1358.

Bulet, P., Dimarcq, J.L., Hetru, C., Lagueux, M., Charlet, M., Hegy, G., Van Dorsselaer, A., and Hoffmann, J.A. (1993). A novel inducible antibacterial peptide of *Drosophila* carries an O-glycosylated substitution. J. Biol. Chem. *268*, 14893–14897.

Cao, Z., Henzel, W.J., and Gao, X. (1996). IRAK: a kinase associated with the interleukin-1 receptor. Science *271*, 1128–1131.

Chasan, N., and Anderson, K.V. (1989). Activation of easter, an apparent serine protease, in organizing the dorsal–ventral axis of the Drosophila embryo. Cell *56*, 391–400.

DeLotto, R., and Spierer, P. (1986). A gene required for the specification of the dorsal-ventral pattern in *Drosophila* appears to encode a serine protease. Nature *323*, 688–692.

Dimarcq, J.L., Hoffmann, D., Meister, M., Bulet, P., Lanot, R., Reichhart, J.M., and Hoffmann, J.A. (1994). Characterization and transcriptional profiles of a *Drosophila* gene encoding an insect defensin: a study in insect immunity. Eur. J. Biochem. *221*, 201–209.

Dinesh-Kumar, S.P., Whitham, S., Choi, D., Hehl, R., Corr, C., and Baker, B. (1995). Transposon tagging of tobacco mosaic virus resistance gene *N*: its possible role in the TMV-*N*-mediated signal transduction pathway. Proc. Natl. Acad. Sci. USA *92*, 4175–4180.

Engström, Y., Kadalayil, L., Sun, S.C., Samakovlis, C., Hultmark, D., and Faye, I. (1993). κB-like motifs regulate the induction of immune genes in *Drosophila*. J. Mol. Biol. *232*, 327–333.

Fehlbaum, P., Bulet, P., Michaut, L., Lagueux, M., Broekaert, W., Hetru, C., and Hoffmann, J.A. (1994). Insect immunity: septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. J. Biol. Chem. *269*, 33159–33163.

Flyg, C., and Boman, H.G. (1988). *Drosophila* genes *cut* and *miniature* are associated with the susceptibility to infection by *Serratia marcescens*. Genet. Res. *52*, 51–56.

Gay, N.J., and Keith, F.J. (1991). *Drosophila Toll* and IL-1 receptor. Nature *351*, 355–356.

Geisler, R., Bergmann, A., Hiromi, Y., and Nüsslein-Volhard, C. (1992). *cactus*, a gene involved in dorsoventral pattern formation of Drosophila, is related to the IκB gene family of vertebrates. Cell *71*, 613–621.

Gertulla, S., Jin, Y., and Anderson, K.V. (1988). Zygotic expression and activity of the *Drosophila Toll* gene, a gene required maternally for embryonic dorsal-ventral pattern formation. Genetics *119*, 123–133.

Gross, I., Georgel, P., Kappler C., Reichhart J.M., and Hoffmann, J.A. (1996). *Drosophila* immunity: a comparative analysis of the Rel proteins dorsal and Dif in the induction of the genes encoding dipteric in and cecropin. Nucl. Acids Res. *24*, 1238–1245.

Hashimoto, C., Hudson, K.L., and Anderson, K.V. (1988). The *Toll* gene of Drosophila, required for dorso–ventral embryonic polarity, appears to encode a transmembrane protein. Cell *52*, 269–279.

Hecht, P.M., and Anderson, K.V. (1992). Extracellular proteases and embryonic pattern formation. Trends Cell Biol. *2*, 197–202.

Hecht, P.M., and Anderson, K.V. (1993). Genetic characterization of *tube* and *pelle* genes required for signaling between *Toll* and *dorsal* in the specification of the dorsal-ventral pattern of the *Drosophila* embryo. Genetics *135*, 405–417.

Heguy, A., Baldari, C.T., Macchia, G., Telford, J.L., and Melli, M. (1992). Amino acids conserved in interleukin-1 receptor (IL-1R) and the *Drosophila Toll* protein are essential for IL-1R signal transduction. J. Biol. Chem. *267*, 2604–2609.

Hetru, C., Bulet, P., Cociancich, S., Dimarq, J.L., Hoffmann, D., and Hoffmann, J.A. (1994). Antibacterial peptides/polypeptides in the insect host defense. In Perspectives in Immunity: The Insect Host Defense, J.A. Hoffmann, C. Janeway, and S. Natori, eds. (Austin, Texas: R.G. Landes Company), pp. 167–181.

Hoffmann, J.A. (1995). Innate immunity of insects. Curr. Opin. Immunol. *7*, 4–10.

Hultmark, D. (1993). Immune reactions in *Drosophila* and other insects: a model for innate immunity. 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). *Dif*, a *dorsal*-related gene that mediates an immune response in Drosophila. Cell *75*, 753–763.

Iwanaga, S. (1993). The limulus clotting reaction. Curr. Opin. Immunol. *5*, 74–82.

Jin, Y., and Anderson, K.V. (1990). Dominant and recessive alleles of the Drosophila *easter* gene are point mutations at conserved sites in the serine protease catalytic domain. Cell *60*, 873–881.

Kappler, C., Meister, M., Lagueux, M., Gateff, E., Hoffmann, J.A., and Reichhart, J.M. (1993). Insect immunity: two 17 bp repeats nesting a κB-related sequence confer inducibility to the diptericin gene and bind a polypeptide in bacteria-challenged *Drosophila*. EMBO J. *12*, 1561–1568.

Kidd, S. (1992). Characterization of the Drosophila *cactus* locus and analysis of interactions between cactus and dorsal proteins. Cell *71*, 623–635.

Kylsten, P., Samakovlis, C., and Hultmark, D. (1990). The cecropin locus in *Drosophila*: a compact gene cluster involved in the response to infection. EMBO J. *9*, 217–224.

Lemaitre, B., Kromer-Metzger, E., Michaut, L., Nicolas, E., Meister, M., Georgel, P., Reichhart, J.M., and Hoffmann, J.A. (1995a). A novel mutation, *immune deficiency*, defines two distinct control pathways in the *Drosophila* host defense. Proc. Natl. Acad. Sci. USA *92*, 9465–9469.

Lemaitre, B., Meister, M., Govind, S., Georgel, P., Steward, R., Reichhart, J.M., and Hoffmann, J.A. (1995b). Functional analysis and regulation of nuclear import of dorsal during the immune response of Drosphila. EMBO J. *14*, 536–545.

Letsou, A., Alexander, S., Orth, K., and Wasserman, S.A. (1991). Genetic and molecular characterization of *tube*, a *Drosophila* gene maternally required for embryonic dorsoventral polarity. Proc. Natl. Acad. Sci. USA *88*, 810–814.

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

Morisato, D., and Anderson, K.V. (1994). The *spätzle* gene encodes a component of the extracellular signaling pathway establishing the dorsoventral pattern of the Drosophila embryo. Cell *76*, 677–688.

Morisato, D., and Anderson, K.V. (1995). Signaling pathways that establish the dorsal-ventral pattern of the *Drosophila* embryo. Annu. Rev. Genet. *29*, 371–399.

O'Connell, P., and Rosbach, M. (1984). Sequence, structure and codon preference of the *Drosophila* ribosomal protein 49 gene. Nucl. Acids Res. *12*, 5495–5513.

Petersen, U.M., Björklund, G., Ip, T.Y., and Engström, Y. (1995). The *dorsal-related* immunity factor, Dif is a sequence-specific transactivator of *Drosophila Cecropin* gene expression. EMBO J. *14*, 3146–3158.

Reichhart, J.M., Georgel, P., Meister, M., Lemaitre, B., Kappler, C., and Hoffmann, J.A. (1993). Expression and nuclear translocation of the *rel*/NF-κB-related morphogen *dorsal* during the immune response of *Drosophila*. CR Acad. Sci. (Paris) *316*, 1207–1217.

Rosetto, M., Engström, Y., Baldari, C.T., Telford, J.L., and Hultmark, D. (1995). Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a *Drosophila* hemocyte cell line. Biochem. Biophys. Res. Commun. *209*, 111–116.

Roth, S., Hiromi, Y., Godt, D., and Nüsslein-Volhard, C. (1991). *cactus*, a maternal gene required for proper formation of the dorsoventral morphogen gradient in *Drosophila* embryos. Development *112*, 371–388.

Schneider, D.S., Jin, Y., Morisato, D., and Anderson, K.V. (1994). A processed form of spätzle protein defines dorso-ventral polarity in the *Drosophila* embryo. Development *120*, 1243–1250.

Shelton, C.A., and Wasserman, S.A. (1993). *pelle* encodes a protein kinase required to establish dorsoventral polarity in the Drosophila embryo. Cell *72*, 515–525.

Siebenlist, U., Franzoso, G., and Brown, K. (1994). Structure, regulation and function of NF-κB. Annu. Rev. Cell Biol. *10*, 405–455.

Smith, C., Giordano, H., and DeLotto, R. (1994). Mutational analysis of the *Drosophila snake* protease: an essential role for domains within the proenzyme polypeptide chain. Genetics *136*, 1355–1365.

Steward, R. (1987). Dorsal, an embryonic polarity gene in *Drosophila* is homologous to the vertebrate proto-oncogene, c-rel. Science *238*, 692–694.

Sun, S.C., Lindström, I., Lee, J.Y., and Faye, I. (1991). Structure and expression of the attacin genes in *Hyalophora cecropia*. Eur. J. Biochem. *196*, 247–254.

Tatei, K., and Levine, M. (1995). Specificity of Rel-inhibitor interactions in *Drosophila* embryos. Mol. Cell. Biol. *15*, 3627–3634.

Tryselius Y., Samakovlis C., Kimbrell, D.A., and Hultmark, D. (1992). *CecC*, a cecropin gene expressed during metamorphosis in *Drosophila* pupae. Eur. J. Biochem. *204*, 395–399.

Vey A., and Götz P. (1986). Antifungal cellular defense mechanisms in insects. In Hemocytic and Humoral Immunity in Athropods, A.P. Gupta, ed. (New York: John Wiley & Sons, Inc.), pp. 89–115.

Wasserman, S.A. (1993). A conserved signal transduction pathway regulating the activity of the Rel-like proteins dorsal and NF-κB. Mol. Biol. Cell *4*, 767–771.

Whitham, S., Dinesh-Kumar, S.P., Choi, D., Hehl, R., Corr, C., and Baker, B. (1994). The product of the tobacco mosaic virus resistance gene *N*: similarity to Toll and the interleukin-1 receptor. Cell *78*, 1101–1115.

Wicker, C., Reichhart, J.M., Hoffmann, D., Hultmark, D., Samakovlis, C., and Hoffmann, J.A. (1990). Insect immunity: characterization of a *Drosophila* cDNA encoding a novel member of the diptericin family of immune peptides. J. Biol. Chem. *265*, 22493–22498.