RESEARCH COMMUNICATION

# Toll receptor-mediated *Drosophila* immune response requires Dif, an NF-κB factor

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**The induction of immunity genes in *Drosophila* has been proposed to be dependent on Dorsal, Dif, and Relish, the NF-κB-related factors. Here we provide genetic evidence that Dif is required for the induction of only a subset of antimicrobial peptide genes. The results show that the presence of Dif without Dorsal is sufficient to mediate the induction of *drosomycin* and *defensin*. We also demonstrate that Dif is a downstream component of the Toll signaling pathway in activating the *drosomycin* expression. These results reveal that individual members of the NF-κB family in *Drosophila* have distinct roles in immunity and development.**

Received November 14, 1998; revised version accepted February 2, 1999.

Multicellular organisms share a common burden of defending themselves against the invasion of microorganisms. Recent molecular genetic studies in plants, insects, and mammals reveal conserved pathways that signal host cells of microbial infection and elicit production of protective molecules (Ip et al. 1993; Whitham et al. 1994; Barillas-Mury et al. 1996; Medzhitov et al. 1997; Ryals et al. 1997; Han et al. 1998; Ip and Davis 1998; Yang et al. 1998). In tobacco, the *N* gene product mediates the resistance to tobacco mosaic virus. The N protein has homology to the intracellular domains of the *Drosophila* Toll and the mammalian interleukin-1 receptor (IL-1R) (Whitham et al. 1994). Such homology is also observed in the *Arabidopsis* RPP5 protein, which confers resistance to downy mildew pathogen (Parker et al. 1997). Using the *Drosophila* Toll sequence, various groups have further identified in human five novel Toll-like receptors (Medzhitov et al. 1997; Rock et al. 1998; Yang et al. 1998). These novel molecules probably represent true homologs of Toll by virtue of having homology in both intracellular and extracellular domains. At least some of these human Toll-like receptors can mediate aspects of immune response (Medzhitov et al. 1997; Rock et al. 1998; Yang et al. 1998). Therefore, the results support the idea that Toll-mediated signaling represents an ancient self-defense pathway. It has also been shown that the stress-activated JNK and p38 MAP kinase pathways, as well as the JAK–STAT pathway, may have similar functions in *Drosophila* (Han et al. 1998; Ip and Davis 1998; Liu et al. 1998; Mathey-Prevot and Perrimon 1998). Taken together, at least some of the pathways that mediate self-defense response in very diverse species are highly conserved.

The activation of Toll and IL-1R both lead to the mobilization of NF-κB factors, which have been shown to be present in many cell types to regulate genes that are involved in self-protection processes (Verma et al. 1995; Baeuerle and Baltimore 1996). In *Drosophila*, the first member of the NF-κB family, Dorsal, was identified in a screen for genes required for embryonic development. Dorsal is a key regulator in determining dorsoventral polarity (Drier and Steward 1997). Both Dorsal and NF-κB can bind to similar DNA sequences and have similar gene regulatory functions. Their activities are also modulated by highly conserved signaling pathways (Verma et al. 1995; Baeuerle and Baltimore 1996; Drier and Steward 1997; Wu and Anderson 1997). Despite the striking similarity of the molecular components involved, the biological processes (dorsoventral development vs. immune response) controlled by Dorsal and NF-κB pathways seemed rather disparate. This disparity, however, was reconciled by the implication of NF-κB-like molecules in regulating *Drosophila* antimicrobial response (Sun and Faye 1992; Engstrom et al. 1993; Ip et al. 1993; Kappler et al. 1993).

Insects battle microbial infection by synthesizing a spectrum of antimicrobial peptides that synergistically lyse invading microorganisms. Whereas >10 different antimicrobial peptides have been identified in different insects, approximately seven genes that encode such peptides have been cloned from *Drosophila* (Hoffmann et al. 1996; Hultmark 1993). Molecular analyses showed that the induction of these peptides in *Drosophila* probably involves NF-κB factors, which include Dif and Relish in addition to Dorsal (Ip et al. 1993; Lemaitre et al. 1995a; Petersen et al. 1995; Dushay et al. 1996; Gross et al. 1996). Therefore, previous reports suggest that the Toll–NF-κB signaling pathway represents an evolutionarily conserved cassette utilized in diverse species in the self-defense process. However, the implication of the insect NF-κB factors in immunity has been based on biochemical and molecular experiments. The understanding of how these molecules may function individually and in combination in whole animal requires further analysis. In this report we present genetic evidence demonstrating that Dif is an essential factor for some aspects of insect immunity.

## Results

To elucidate the requirements of NF-κB-related factors in *Drosophila* immunity, we attempted to isolate mutants that were defective in Dif and Dorsal function. *Dif*

[*Key Words*: Immunity; *Drosophila*; Dif; NF-κB; Toll; drosomycin]
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was mapped previously near the *dorsal* locus (Ip et al. 1993). Molecular analysis revealed that the transcription start site of the *Dif* gene is ~9 kb distant from the 3′ end of *dorsal* (Fig. 1). The homology of the Rel domains (Ip et al. 1993) and the similarity of the intron–exon structures (Fig. 1) suggest that the two genes might arise by duplication during evolution. Previous results showed that *Dif* is expressed at high levels at postembryonic stages, whereas *dorsal* has high maternal and low zygotic expression (Steward et al. 1984; Ip et al. 1993; Lemaitre et al. 1995a). Nonetheless, it has been demonstrated that *Dif* can rescue to some extent the *dorsal* mutant phenotype in the early embryo (Stein et al. 1998). The two transcription factors, therefore, can regulate similar target genes if expressed in the same tissue. Previous reports showed that Dif and Dorsal are present in fat bodies and hemocytes, the insect immune organs (Ip et al. 1993; Lemaitre et al. 1995a, 1996; Petersen et al. 1995; Gross et al. 1996). Moreover, both proteins accumulate in nuclei upon microbial infection (Ip et al. 1993; Lemaitre et al. 1995a; Petersen et al. 1995). Although the induction of immunity genes is normal in *dorsal* mutants (Lemaitre et al. 1995a, 1996), molecular and biochemical observations raise the possibility that the two factors perform redundant functions during the immune response. Therefore, genetic approach should help to differentiate the roles of these two regulators in vivo.

We employed the strategy of local *P* element hopping (Tower et al. 1993) to try to isolate insertional mutations of *Dif*. Because the *Dif* gene is located close to *dorsal*, we used a *P* element strain (P01313) that is allelic to *dorsal* mutants (Berg and Spradling 1991) and has an insertion mapped to *dorsal* (Fig. 1). After screening >3000 lines, no placement of the *P* element into the *Dif* gene was obtained. Instead, we isolated a new insertion (P1522) into a neighboring transcription unit that we named *C2* (Fig. 1). *C2* is expressed in the CNS of the developing embryo and encodes a putative protein that has only short stretches of homology with a *Saccharomyces cerevisiae* gene and a *Caenorhabditis elegans* gene with no known function (data not shown). We further attempted to mobilize P1522, which is closer to *Dif*, to isolate an insertion in the *Dif* locus, but without success. Therefore, we isolated fly lines that might contain deletion in the region by imprecise excision of the P1522 *P* element. Using this approach, we obtained a deletion strain that we called *J4*. Molecular mapping by Southern blots using *dorsal*, *Dif*, *C2*, and *Bicaudal D* cDNA revealed that the *J4* deletion uncovered a region extending from the promoter of *dorsal* to the promoter of *C2* (Fig. 1). We surmise that the deletion might have arisen from the recombination of the *P* element located on the *C2* promoter and some remnant *P*-element sequence from the parent P01313 line. Greater than 99% of the *J4* homozygous flies do not survive to adulthood, although we have not tested whether the lethality is associated with the deletion or with other mutations on the chromosome. However, a few homozygous adult escapers can be found consistently. These survivors have no obvious morphological abnormality. We isolated RNA from the homozygous flies and performed Northern analysis. The results in Figure 2 demonstrated that no RNA expression of *Dif*, *dorsal*, or *C2* was detected, consistent with the molecular mapping data that these three genes are deleted. Therefore, the *J4* deletion represents a null mutation of both *Dif* and *dorsal*.

Homozygous *J4* flies were collected and used for immunity gene induction experiments. The flies were injected with needles previously dipped into saturated *Escherichia coli* cultures. The infected flies were allowed to recover for 3 or 6 hr. RNAs were isolated and analyzed by Northern blots. We used five antimicrobial peptide gene cDNAs as probes to assess the induction of the endogenous genes in the infected flies. These genes exhibit different induction kinetics (Fig. 3) (Hoffmann et al. 1996). For instance, *cecropin* is induced to high levels at 3 hr and the level has declined after 6 hr. *drosomycin* expression, on the other hand, increases steadily at 3–6 hr after induction. In the parental $dorsal^{P01313}$ mutant strain, the induction of all five genes were similar to that of wild-type flies, as reported previously (Lemaitre et al. 1995a, 1996; Petersen et al. 1995; also see Fig. 4). The *J4* deletion, however, exhibited specific defects in the induction of *drosomycin* and *defensin* (Fig. 3A,B). In contrast, induction of *cecropin*, *attacin*, and *diptericin* is not affected in the *J4* mutant. These results demonstrate that *Dif* is required specifically for the induction of only a subset of immunity genes. Although the *dorsal* mutation alone does not affect *drosomycin* and *defensin* induction, as *J4* is a *Dif dorsal* double mutant the results presented so far cannot exclude the possibility that the two regulators have redundant functions.

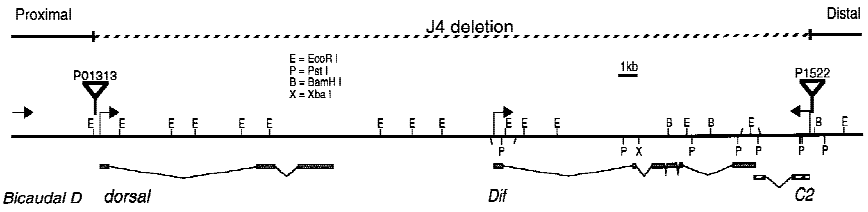


**Figure 1.** Restriction map and intron–exon structure of the *Dif–dorsal* locus, which includes *dorsal*, *Dif* and *C2*. *Bicaudal D* is located ~5 kb upstream of *dorsal* (Wharton and Struhl 1989). Arrows indicate the start sites and directions of transcription, except the arrow on *Bicaudal D* shows only the direction of transcription. The exons are represented by rectangular boxes; the introns are represented by the angled lines. All the known *Eco*RI restriction sites are shown; *Pst*I, *Bam*HI, and *Xba*I sites are shown only on the *Dif* and *C2* genes. The *Eco*RI restriction map of the *dorsal* gene is duplicated from Steward (1987). *P*-element insertions are indicated by flags. The deletion in the *J4* chromosome (broken line), uncovers the three genes but does not affect *Bicaudal D*.

To distinguish the roles of *Dif* and *dorsal* in the immune process, we performed a genetic rescue experiment. Rescue plasmids that utilized the *tubulinα1* promoter (Basler and Struhl 1994) to direct a ubiquitous expression of *Dif* or *dorsal* were constructed. Transgenic flies were ob-

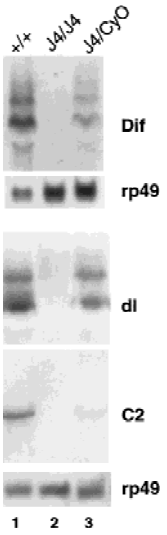


**Figure 2.** Northern analysis of the expression of the *Dif–dorsal* locus in the *J4* deletion. Total RNAs were isolated from Canton-S wild-type flies (+/+ , lane *1*), *J4* homozygous flies (*J4/J4*, lane *2*), or heterozygous *J4* flies (*J4/CyO*, lane *3*). RNAs were analyzed on denaturing gels, transferred to membranes, and hybridized with radiolabeled probes as indicated (*right*). (*dl*) *dorsal*. The ribosomal protein gene *rp49* was used as a loading control.

tained for both constructs, and the chromosomal locations of the transgenes were determined by standard genetic crosses. The chromosomes that contained the rescue transgenes were crossed or recombined together with the *J4* chromosome. Stable lines were established, and the induction of *drosomycin* and *defensin* was examined. The results demonstrate that *Dif* can rescue the induction of the antimicrobial peptide genes, whereas *dorsal* cannot (Fig. 4A,B). We have analyzed the RNA expression of the transgenes, and the results show that *tubulin–dorsal* is expressed to a level higher than the endogenous *dorsal* (Fig. 4C). To ascertain that the lack of rescue by *dorsal* was not due to the production of a nonfunctional protein, we crossed the *tubulin–dorsal* transgene into a well-characterized *dorsal* mutant background to test for the function. The *dorsal*$^{15}$ allele is a severe mutation, and embryos derived from homozygous mothers exhibit completely dorsalized cuticle phenotype (Fig. 4D, left). The homozygous mothers that also carried the *tubulin–dorsal* transgene, on the other hand, produced embryos that had well defined dorsoventral polarity and ventral denticle belts (Fig. 4D, right). The *tubulin–Dif* transgene expressed a relatively lower level of mRNA in adults and could nonetheless rescue the embryonic dorsoventral developmental defect (data not shown). Therefore, we conclude that the lack of rescue of the immune response by *tubulin–dorsal* is not due to the absence of expression of functional dorsal protein. Instead, it indicates that *Dif* is sufficient to mediate the induction of *drosomycin* and *defensin*, and that *dorsal* cannot substitute the role of *Dif*.

Previous experiments demonstrated that in the gain-of-function *Toll*$^{10b}$ mutant background, *drosomycin* is constitutively expressed at high levels, suggesting that the Toll signaling pathway regulates the antifungal gene expression during infection (Lemaitre et al. 1996). This notion was supported further by the analyses of loss-of-function mutants of the *Toll* signaling pathway components (Lemaitre et al. 1996; Nicolas et al. 1998). Although Dorsal functions downstream of Toll during dorsoventral patterning in the early embryo, it is not clear which of the NF-κB-related proteins are employed in the Toll mediated immune response (Petersen et al. 1995; Gross et al. 1996; Lemaitre et al. 1996;). We carried out a genetic experiment to test whether Dif acts downstream of Toll in regulating *drosomycin* gene expression. The *J4* and *dorsal* loss-of-function mutants were crossed with the *Toll*$^{10b}$ gain-of-function mutant. The flies that contained different combinations of marker chromosomes were collected and analyzed for the expression of *drosomycin* (Fig. 5). In wild-type flies, *drosomycin* was expressed at a basal level (lane 2), and the expression was much elevated in the *Toll*$^{10b}$ flies (lane 1). This *Toll*$^{10b}$

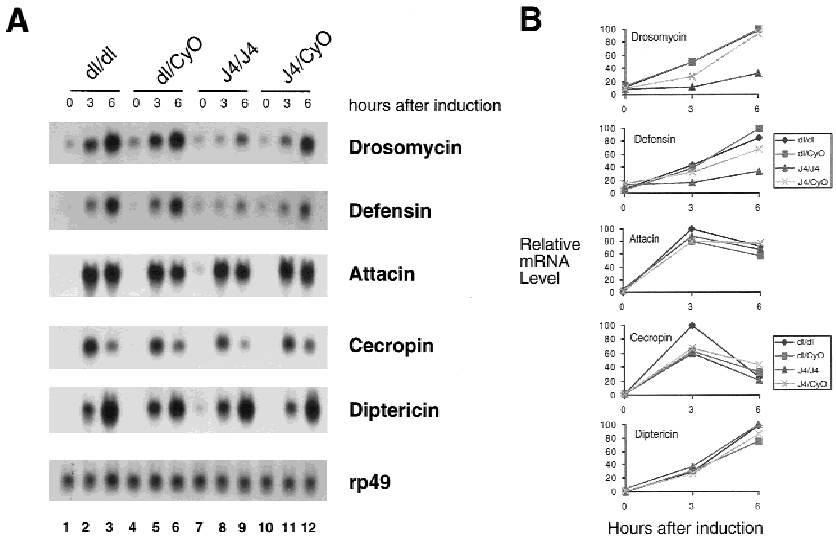


**Figure 3.** Induction of antimicrobial peptide genes in the *Dif/dorsal* deletion mutant. (*A*) Total RNAs were isolated from fly strains with the genotype indicated at *top*. The *dorsal* (*dl*) mutant used in this and other experiments was the parental P01313 line. Prior to RNA isolation, the flies were induced by injection of *E. coli* bacteria and allowed to recover for 3 or 6 hr. The 0-hour represents no injection. The isolated RNAs were analyzed by Northern blot and hybridized with radiolabeled probes indicated at *right*. Each panel utilized the same set of RNA isolated in parallel, but the times of exposure varied from 12 to 72 hr. This is a representative result of three independent experiments. (*B*) Quantitative analysis of gene induction. The hybridization signal of the experiments shown in *A* was quantitated using PhosphorImager (Molecular Dynamics). The relative mRNA levels, normalized with the *rp49* signal, were plotted as shown. The value of 100 was assigned to the highest signals of the individual blots; the other signals were calculated as a fraction of this value.

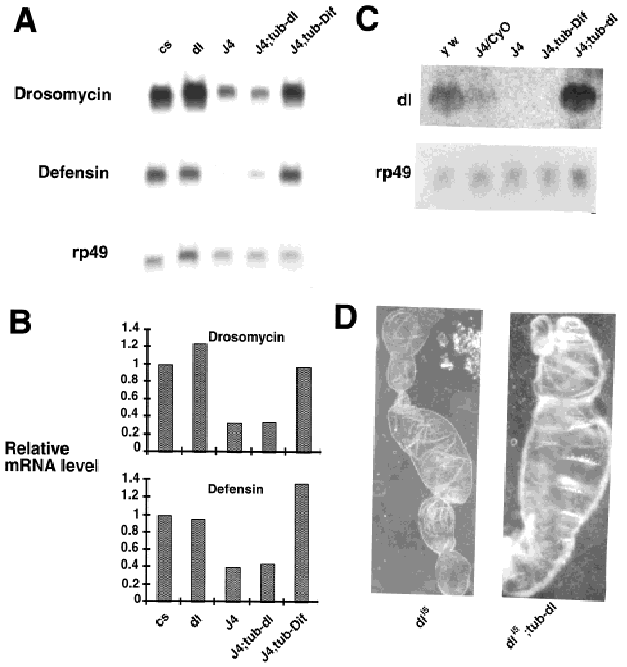


**Figure 4.** Ubiquitous expression of *Dif* rescues the defect of immune response. (*A*) Transgenic flies containing the *tubulinα1–dorsal* or *-Dif* constructs were crossed or recombined, respectively, with the *J4* chromosome. Flies that had the genotypes indicated were induced by bacterial injection and after 6 hr the RNA were isolated for Northern analysis. The autoradiographs of the blots hybridized with the indicated probes are shown. (*B*) The blots were quantitated using PhosphorImager, and the *drosomycin* and *defensin* signals were normalized with that of *rp49*. The relative levels of mRNA expression were plotted, with the Canton S (cs) wild-type fly expression levels assigned as 1. The graphs show the average result of two independent experiments. (*C*) Control experiments were carried out using the transgenic lines to examine the expression of *dorsal* in adult flies. The *tubulin–dorsal* transgene drives the expression of *dorsal* mRNA to a level higher than that in the parental *y w* flies. (*D*) The severe cuticle phenotype exhibited in embryos derived from $dorsal^{15}$ mutant mothers (*left*) could be rescued by the *tubulin-dorsal* transgene (*right*), demonstrating that the transgene can produce functional Dorsal proteins.

activated expression of *drosomycin* was clearly suppressed by the homozygous *J4* chromosome (lane 3). Other mutants or marker chromosomes in the $Toll^{10b}$ background did not cause any significant decrease of the constitutive, high-level expression of *drosomycin*. Because the *dorsal* mutation itself cannot suppress the $Toll^{10b}$ effect (lane 7), the results demonstrate that Dif is an essential component of the Toll signaling pathway in the induction of *drosomycin*. We have not, however, ruled out the possibility that dorsal can replace the function of Dif in Toll signaling because of the double deletion in the *J4* chromosome. Nevertheless, in consideration of the rescue experiments presented above, there is no indication that Dorsal performs essential function downstream of Toll during the immune response.

## Discussion

The presented results demonstrate that *Dif* is an essential component of the insect immune response. These results corroborate with recent evidence suggesting that diverse species utilize similar molecules to combat microbial infection (Hoffmann et al. 1996; Wilson et al. 1997; Han et al. 1998; Ip and Davis 1998; Wu and Anderson 1998). *Toll* and related proteins are present in humans, fruit flies, and tobacco and have been shown to be involved in transmitting signals provoked by infection (Whitham et al. 1994; Lemaitre et al. 1996; Medzhitov et al. 1997; Parker et al. 1997; Williams et al. 1997; Chaudhary et al. 1998; Rock et al. 1998; Yang et al. 1998). Previous reports revealed that in *Drosophila* the *Toll* pathway is essential for the induction of the antifungal peptide gene *drosomycin* and may participate in the induction of some other antibacterial peptide genes (Lemaitre et al. 1996; Wu and Anderson 1998). However, the transcription factor that mediates the *Toll* response has not been identified unambiguously. Our genetic experiments demonstrate that *Dif* acts downstream in the *Toll* signaling pathway for the induction of *drosomycin*. Furthermore, loss-of-function of *Dif* causes an impairment in the induction of *drosomycin* as well as *defensin*. However, *defensin* expression is not significantly elevated in the $Toll^{10b}$ mutant (Lemaitre et al. 1996; data not shown). It may be that the activation of Dif is necessary but not sufficient to induce *defensin*. Such induction may also require other factors that are activated by normal immune challenge but are independent of the Toll pathway.

Earlier results and our experiments do not suggest an essential function of *dorsal* during immune response. However, it is possible that Dif/Dorsal heterodimer can mediate some in vivo regulation. It is also plausible that some immunity genes that are yet to be identified require *dorsal*. Because the induction of at least three other antibacterial peptide genes, that is, *diptericin*, *cecropin*, and *attacin*, is not affected in the *Dif dorsal* double mutant, this indicates that some other NF-κB-related factors, such as Relish, may be involved in regulating the

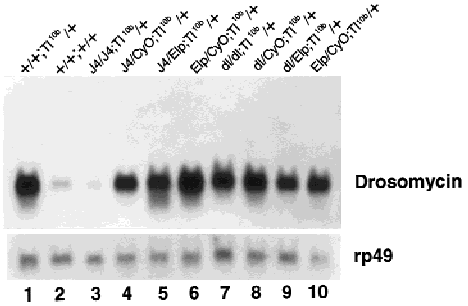


**Figure 5.** Dif is a downstream component of the Toll signaling pathway. $Toll^{10b}$ gain-of-function mutant male flies were mated with *yw; BcElp/CyO; Ki/TM6y*$^+$ double balancer flies. Similarly, the *J4/CyO* or $dl^{01313}$*/CyO* flies were mated with double balancer flies. $F_1$ flies with the correct markers were mated together to generate the $F_2$ flies (genotype shown above lanes *3–10*). The flies were used directly without bacterial injection for RNA isolation and Northern analysis. An autoradiograph after hybridization with the *drosomycin* probe is shown.

repertoire of immunity genes that contain the κB element (Lemaitre et al. 1995a, 1996; Petersen et al. 1995; Dushay et al. 1996; Gross et al. 1996). Gene knockout experiments in mice revealed that during immune response the mice bearing individual loss-of-function mutations of NF-κB show different defects (Baeuerle and Baltimore 1996; Attar et al. 1997). Our results with *Drosophila* are reminiscent of these specific requirements of the NF-κB factors in different aspects of self-defense response.

A previous report demonstrated that 18-Wheeler (18W), a member of the Toll and IL-1R family, has a critical function in the *Drosophila* immune response (Williams et al. 1997). In *18w* mutants, the nuclear localization of *Dif* is blocked, whereas that of *dorsal* is normal. Furthermore, *attacin* expression is significantly affected, but that of *diptericin* is normal. These results suggest that 18W activates Dif, which in turn mediates the induction of *attacin*. Mutational analysis reported here, however, shows that the *attacin* induction is not affected in the absence of Dif. It is possible that Dif is only one of the components that functions downstream of 18W. Other members of the family, such as Relish, may be able to substitute Dif in the activation of *attacin*.

Because Dif localization was not affected in Toll loss-of-function mutations, it was proposed that Toll utilized Dorsal,wherease 18W utilized Dif to mediate some aspects of the immune response (Wu and Anderson 1998). The results presented here suggest that Dif function is required for the activated Toll receptor to induce *drosomycin* gene expression. Moreover, it has been demonstrated that in $Toll^{10b}$ larvae there are elevated levels of nuclear and cytoplasmic Dif in the fat bodies (Ip et al. 1993). Therefore, at least under some circumstances, Toll utilizes Dif, or Dif/Rel heterodimer where Rel is any other member of the family, as the transcription factor. It is possible that both 18W and Toll can modulate Dif activity, which explains the results that in *Toll* mutants Dif can translocate to nucleus, as 18W responses to the immune challenge. On the other hand, in *18w* mutants, there may still be residual nuclear Dif activity (Williams et al. 1997), probably due to the activation of Toll.

In addition to Toll and 18W, other components may be used to regulate the activity of NF-κB proteins in *Drosophila*, as genetic screens have identified a number of mutations that are essential for the activation of *diptericin* and for the regulation of NF-κB proteins (Lemaitre et al. 1995b; Wu and Anderson 1998). These mutations constitute multiple pathways, which differentially regulate the nuclear localization of Dif and Dorsal. Taken together, the data suggest that although some components may have critical roles in regulating specific immunity genes, such as the control of *drosomycin* by the Toll/Dif pathway, a cross-regulatory network is likely present in inducing the expression of multiple immunity genes.

## Materials and methods

Drosophila *genetics*

Flies were kept in standard yeast/agar/molasses/cornmeal medium. For *P* element mobilization, the female flies with the *P* element insertion $ry^+$ were mated with male flies with the genotype *w*; +/+; *TM3,Sb,Δ2-3*/*Ubx,Δ2-3*. Male flies of the $F_1$ generation were mated with *BcElp*/*CyO*; *ry* female balancer flies. $F_2$ males that did not carry Δ2-3 marker chromosomes but expressed $ry^+$ and *CyO* markers were collected and mated batch-wise with the same balancer flies. After 3–5 days, the male flies were separated and used for DNA isolation and inverse PCR reaction to determine the insertion into the *Dif*/*C2* locus. Individual males of the $F_3$ generation were used to mate with balancer flies to establish stocks and were rescreened for insertion. The imprecise excision of the *P* element was performed similarly, except that individual *ry* flies were collected after exposure to *Δ2-3*. DNAs isolated from the established lines were used for genomic Southern analysis to determine the integrity of the *Dif*/*dl* locus. Cuticle preparations were carried out by first collecting embryos that were aged for ~24 hr. The embryos were dechorionated and incubated in 85% lactic acid at 70°C for 12 hr. Cuticle preparations were photographed using dark-field microscopy.

*Molecular analysis*

For genomic DNA isolation, ~25 flies were homogenized with a handheld motorized pestle. The homogenates were extracted with phenol–chloroform organic solvents and precipitated with ethanol. The genomic DNAs were digested with appropriate restriction enzymes for Southern blot analysis or digested with restriction enzymes and then self-ligated for inverse PCR reaction. For RNA isolation, ~25 flies were homogenized in a buffer containing 50 mM sodium acetate (pH 5.2), 10 mM EDTA, and 1% SDS. Equal volumes of phenol equilibrated with 50 mM sodium acetate (pH 5.2) and 10 mM EDTA were added, and the mixed homogenates were incubated at 65°C for 5 min. The phenol extraction and 65°C incubation were repeated once, and the samples were extracted with phenol–chloroform and precipitated with ethanol. About 40 mg of total RNA was analyzed by formaldehyde–agarose gel electrophoresis. The separated RNAs were blotted onto GeneScreen Plus membrane (NEN) and hybridized with radiolabeled probes as described previously (Ip et al. 1993).

The construction of *Dif* and *dorsal* rescue plasmids was performed by PCR amplification of the corresponding cDNA. The PCR products were digested with *Kpn*I and *Xba*I; these restriction enzyme sites were introduced through the PCR primers. The digested products were then purified and cloned into the pCaSpeR–*tubulinα1* vector digested with the same restriction enzymes. The vector contains ~2.6 kb of the *tubulina1* promoter, and an SV40 3′ sequence (Basler and Struhl 1994). The rescue plasmids were injected into embryos of *y w* flies together with the *Δ2-3* helper plasmid to generate germ-line transformants.

## Acknowledgments

We thank Roger Davis and Li Zeng for helpful comments on the manuscript. We are indebted to Christine Rushlow for providing part of the *Dif* genomic sequence, Allan Spradling for the *P*-element lines, Jin Jiang, Robin Wharton, Dan Hultmark, and Jules Hoffmann for plasmid DNA, and Ruth Steward and Shubha Govind for communicating unpublished results. This work was supported by a grant from the National Institutes of Health (GM53269) and by a Scholar Award of the Leukemia Society of America (to Y.T.I.).

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