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# The *18-wheeler* mutation reveals complex antibacterial gene regulation in *Drosophila* host defense

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**Mammals and insects employ similar Rel/NF-κB signaling cascades in their humoral immune responses. The mammalian interleukin-1 type I receptor (IL-1R) is one way of activating this cascade. The *Drosophila* Toll protein, whose cytoplasmic domain shows striking similarity to that of the IL-1R, acts in the humoral antimicrobial response. Here we demonstrate that a second IL-1R-related *Drosophila* protein, 18-Wheeler (18W), is a critical component of the humoral immune response. *18-wheeler* is expressed in the larval fat body, the primary organ of antimicrobial peptide synthesis. In the absence of the 18W receptor, larvae are more susceptible to bacterial infection. Nuclear translocation of the Rel protein Dorsal-like immunity factor (Dif) is inhibited, though nuclear translocation of another Rel protein, Dorsal, is unaffected. Induction of several antibacterial genes is reduced following infection, relative to wild-type: *attacin* is reduced by 95%, *cecropin* by 65% and *diptericin* by 12%. Finally, *18-wheeler* (*18w*) expression is induced in response to infection and, in addition to the receptor form, four immune-specific transcripts and proteins are produced.**

*Keywords*: *Drosophila*/fat body/insect immunity/IL-1 receptor/lymph gland/Rel-related protein

## Introduction

Insects respond to infection with antimicrobial peptides rapidly produced by the fat body and hemocytes. Pioneering work was carried out in larger insects, but *Drosophila*, with its genetic and molecular advantages, is the insect in which the humoral response is best understood (Hultmark, 1993; Cociancich *et al.*, 1994; Hoffmann, 1995). The emerging paradigm implicates a Rel/NF-κB cascade, analogous to that in mammalian innate immunity and in dorsal–ventral patterning in the *Drosophila* embryo (Hultmark, 1993). Three Rel domain proteins, Dorsal, Dif and Relish, are present in the fat body of larvae and adults and are induced in response to infection (Petersen *et al.*, 1995; Dushay *et al.*, 1996; Lemaitre *et al.*, 1996). There are multiple κB sites in the regulatory regions of the antimicrobial peptide genes (Engström *et al.*, 1993; Georgel *et al.*, 1993).

Mammalian NF-κB is a homo- or heterodimer of Rel proteins. While Dorsal has been shown to act as a homodimer during embryonic axis formation (Govind *et al.*, 1992), the potential exists for Rel protein heterodimers to be active in immunity (Gross *et al.*, 1996). All three Rel proteins have been shown to promote antimicrobial peptide synthesis in cell transfection assays (Engström *et al.*, 1993; Petersen *et al.*, 1995; Dushay *et al.*, 1996; Gross *et al.*, 1996). However, relatively little is known of the receptors involved in initiating the cascade. The Rel/NF-κB paradigm predicts that Toll or another receptor with an interleukin-1 receptor (IL-1R) domain will activate signaling. The maternal role of Toll in establishing embryonic dorsal–ventral polarity is well established. Active Spätzle ligand is produced in a spatially restricted series of proteolytic steps. It binds the Toll receptor which, through the activities of Tube and Pelle, results in degradation of the IκB protein Cactus and release of the Rel protein Dorsal. Dorsal then moves to the nucleus, where it acts as a transcription factor (reviewed by Chasan and Anderson, 1993; Belvin and Anderson, 1996). A zygotic role for the dorsal–ventral cascade genes in immunity has recently been tested (Lemaitre *et al.*, 1996). Mutations in several members of the cascade show similar but selective effects on the ability of the fly to produce antimicrobial peptides. These results suggest that much of the Toll signaling cascade acts as a functional unit in the immune response and they further imply that other receptors are required to activate the full complement of immune responsive genes.

We have previously cloned a *Drosophila* gene, *18-wheeler* (*18w*), with similarity to Toll (Eldon *et al.*, 1994). The extracellular regions of Toll and 18W have two domains containing multiple leucine-rich repeats with N- and C-terminal cysteine-rich flanking domains. Following a single membrane spanning region they share ~200 amino acids of sequence similarity with the cytoplasmic domain of the type I IL-1R. C-Terminal to the IL-1R domain sequence conservation is lost and each has unique sequence.

Here we demonstrate that *18w* is an essential receptor for the *Drosophila* humoral immune response. It has been previously demonstrated that *18w* encodes a protein with receptor-like structure (Chiang and Beachy, 1994; Eldon *et al.*, 1994). We now show that it is expressed in the fat body at the appropriate time to be active in the larval antimicrobial response. *18w* mutant flies show increased lethality in the face of bacterial challenge. Consistent with its sequence similarity to the cytoplasmic domain of type I IL-1Rs, *18w* plays a role in nuclear recruitment of the Rel factor Dif to the nucleus. As is seen in most, if not all, genes shown to be involved in the immune response,

*18w* is transcriptionally induced upon infection. In addition to the expected 5.6 kb transcript previously characterized, four novel transcripts accumulate after infection. Finally, analysis of *18w* mutants reveals alterations in antimicrobial gene expression following infection. A major reduction is seen in *attacin* and *cecropin* synthesis and a minor reduction is seen in *diptericin* synthesis. Our data support the emerging model that *Drosophila* antimicrobial response is regulated in a complex manner.

## Results

*18w* shares significant sequence similarity with *Toll* (Chiang and Beachy, 1994; Eldon *et al.*, 1994), though components of the *18w* signaling pathway have not been identified. *18w* is expressed widely in the embryo and in the imaginal discs of the larvae (Chiang and Beachy, 1994; Eldon *et al.*, 1994), but homozygous hypomorphic mutants usually die during larval development with no obvious cuticular phenotype (Eldon, unpublished observations). Since homozygous larvae are recovered at higher frequency than homozygous adults, all the studies presented here were carried out using third instar larvae.

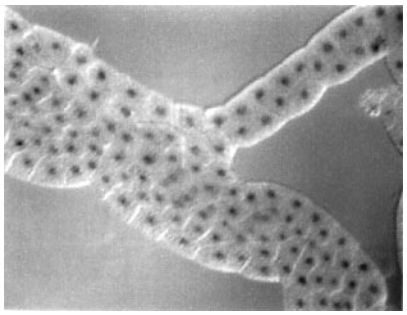

**Fig. 1.** β-Galactosidase expression in heterozygous l(2)00053 third instar larvae and *in situ* analysis in Oregon$^R$ third instar larvae. (**A**) Enhancer detector strain l(2)00053 heterozygotes show staining for β-galactosidase activity in the third instar larval fat body. All staining is seen in the nuclei due to the presence of a nuclear localization signal in the *PZ* construct (Mlodzik and Hiromi, 1992). The P element is inserted ~400 bp 5′ of the *18w* ORF. (**B**) Tissue *in situ* hybridization to wild-type third instar larvae using digoxygenin-labeled *18w* cDNA shows *18w* transcript localization in the fat body (fb) and some staining in the salivary gland (sg).

### *Larval expression of 18-wheeler*

Expression of the β-galactosidase reporter gene by the enhancer detector strain l(2)00053 (Karpen and Spradling, 1992) has been shown to reflect expression of *18w* accurately in embryos (Chiang and Beachy, 1994; Eldon *et al.*, 1994). Heterozygous larvae from this strain were used to determine whether *18w* is expressed in tissues known to play a role in the immune response. Figure 1 demonstrates that β-galactosidase accumulates in the fat body. To test that *18w* transcript accumulated in the fat body, *in situ* hybridization was performed on wild-type third instar wandering larvae. *18w* transcript was detected in the fat body and some staining was seen in the salivary glands (data not shown). Accumulation of β-galactosidase and *18w* transcript in larval fat body is consistent with an immune role for *18w.* Fat body is the primary site of antimicrobial peptide synthesis and secretion (Hultmark, 1993; Cociancich *et al.*, 1994; Hoffmann, 1995). The low level of transcription seen in the salivary gland was not unexpected, since *18w* expression is seen there during embryonic development.

To analyze the distribution of 18W protein in the fat body, immunohistochemical localization to dissected tissues was performed using affinity-purified polyclonal antibodies directed against a bacterially expressed portion of the cytoplasmic domain of the 18W protein (see Materials and methods). Figure 2 compares 18W protein expression in wild-type larvae with homozygous *18w*$^{7–35}$ mutant larvae. The *18w*$^{7–35}$ mutation is caused by a 2.2 kb deletion induced by inaccurate excision of the *PZ* element in l(2)00053. The N-terminal 1.7 kb of the open reading frame is missing in these mutants (Eldon *et al.*, 1994). 18W protein was detected in wild-type fat body (Figure 2A) and its localization to the plasma membrane (arrows, Figure 2B) is consistent with the predicted protein structure. Note that staining was seen on the apical, lateral and basal membranes of these cells (Figure 2B). 18W protein was not restricted to the membrane and was also detected in the cytoplasm of fat body cells (Figure 2A). In *18w*$^{7–35}$ larvae 18W protein was detected only in the cytoplasm, suggesting that this deletion mutant produces a truncated form of 18W protein (see below). To confirm that plasma membrane staining was absent in *18w*$^{7–35}$ larvae (arrows Figure 2C) the image in Figure 2C was photographed at a higher magnification than that in Figure

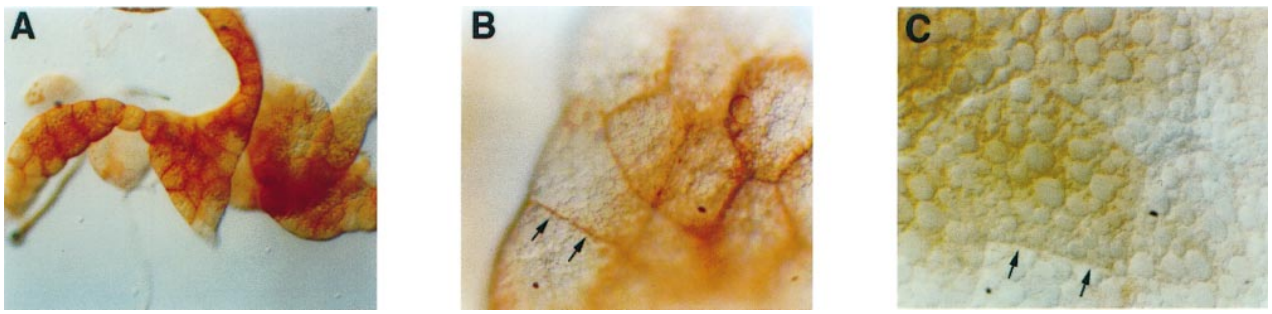


**Fig. 2.** 18-Wheeler protein expression in Oregon$^R$ and homozygous *18w*$^{7–35}$ third larval instar fat body. Oregon$^R$ (A and B) and *18w*$^{7–35}$ (C) fat body was stained with anti-18W antiserum and a HRP-conjugated secondary antibody. (**A**) Low magnification (10× objective) of Oregon$^R$ fat body. Staining can be seen throughout the fat body cells with the most intense staining seen at the plasma membranes. (**B**) Higher magnification (20× objective) view of Oregon$^R$ fat body. 18W protein is enriched at the plasma membranes (arrows), with lighter staining seen in the cytoplasm. (**C**) High magnification (40× objective) of *18w*$^{7–35}$ fat body. There is no 18W protein at the plasma membrane (arrows), while the cytoplasmic staining remains.

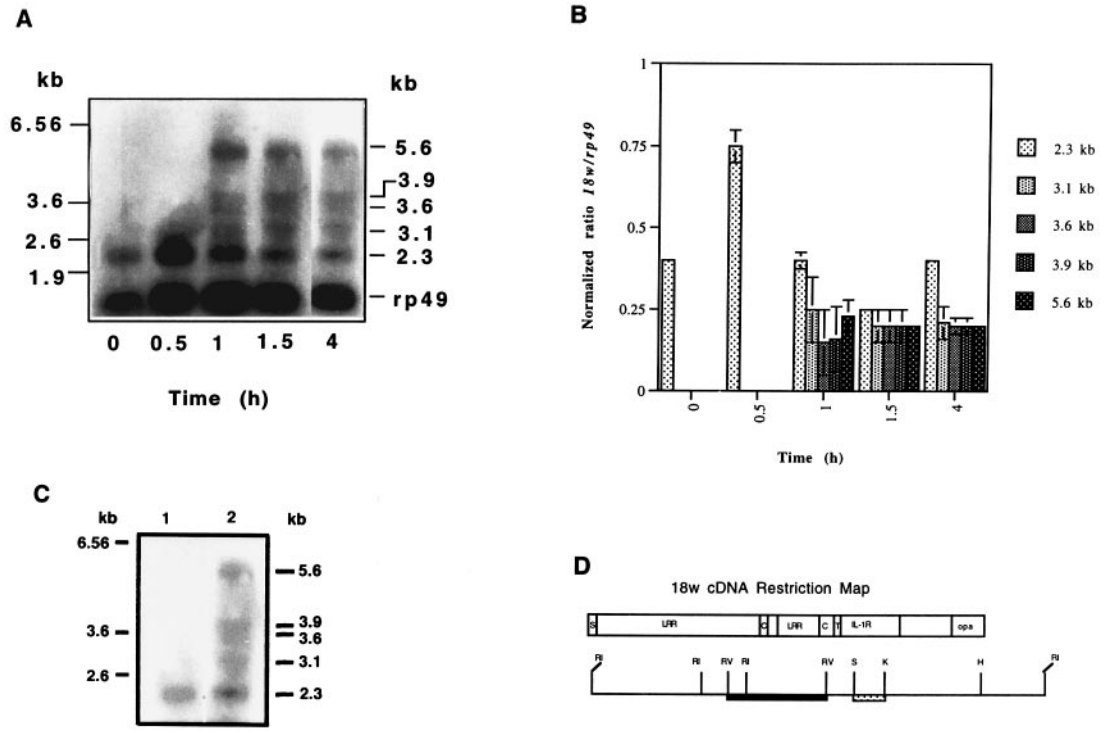


**Fig. 3.** Time course analysis of *18w* transcription in Oregon$^R$ and *18w*$^{7–35}$ mutant larvae. (**A**) Northern analysis of *18w* transcript accumulation in Oregon$^R$ larvae infected with log phase *E.coli* at the wild-type $LD_{40}$. Total RNA was collected at different times after infection. The sizes of the novel transcripts and location of the *ribosomal protein 49* (*rp49*) transcript are shown on the right, while migration of the molecular weight standards is indicated on the left. Numbers at the bottom indicate time elapsed after infection, though 0 indicates uninfected larvae. (**B**) Levels of each of the *18w* transcripts were measured densitometrically using an Ultrascan XL laser densitometer and corrected for RNA loading based on the *rp49* signal. Transcript induction profiles are shown with their respective confidence levels. (**C**) Analysis of *18w* transcript levels in *18w*$^{7–35}$ homozygous mutants (lane 1) and Oregon$^R$ (lane 2) larval RNA collected 1 h after infection with *E.coli*. The sizes of the transcripts and migration of the molecular weight standards are indicated as above. (**D**) Restriction map of the *18w* cDNA corresponding to the ORF. S, signal sequence; LRR, leucine-rich repeats; C, cysteine-rich motif; T, transmembrane domain; IL-1R, interleukin-1 receptor type I cytoplasmic domain; opa, glutamine-rich repeat. The 1.1 kb *Eco*RV restriction fragment was used as probe in (A) and the 300 bp *Sal*I–*Kpn*I restriction fragment was used as probe in (C).

2B. 18W protein was also seen in the lymph gland and garland cells (data not shown). Lymph glands are the organs of hematopoiesis and blood cells are sites of antimicrobial peptide synthesis and secretion as well as being active in coagulation, encapsulation and phagocytosis (reviewed by Rizki, 1978). The garland cells are thought to be involved in removal of toxic substances from the hemolymph (reviewed by Rizki, 1978) and have been shown to be sites of antimicrobial peptide synthesis in *Manduca sexta* (Dickinson *et al.*, 1988). We conclude that 18W protein is present in the appropriate tissues at the appropriate time to function as a receptor in response to infection, and that the *18w*$^{7–35}$ mutant lacks the receptor form of the 18W protein.

### *Induction of 18w transcript*

A hallmark of genes involved in the immune response, including *Toll*, is that their expression is induced following bacterial challenge (Åsling *et al.*, 1995; Dushay *et al.*, 1996; Lemaitre *et al.*, 1996). To determine whether expression of *18w* is affected by infection, RNA was isolated from wandering third instar Oregon$^R$ larvae prior to bacterial challenge and at intervals thereafter. *18w* transcript levels were compared with those of an internal control (Figure 3A and B). Two striking observations were made. First, *18w* transcript levels increased following infection. More surprisingly, novel forms of *18w* transcripts were detected in addition to the expected 5.6 kb transcript whose expression pattern we have previously characterized (Eldon *et al.*, 1994). These novel transcripts had apparent lengths of 2.3, 3.1, 3.6 and 3.9 kb (Figure 3A). The 2.3 kb transcript was the only one readily detected prior to infection, though with significantly longer exposure times the 5.6 kb transcript can also be detected. The 2.3 kb transcript was more rapidly induced than the others. It reached maximal accumulation 30 min after infection, while the other transcripts accumulated maximally 1 h after infection (Figure 3A and B). All five *18w* transcripts were detected with a probe encoding a membrane-proximal region of the extracellular domain and with a probe encoding a portion of the cytoplasmic IL-1R domain (Figure 3D). The abundant presence of a previously undetected transcript was of some concern and we are currently investigating it in more detail. One likely explanation is that in our earlier studies RNA was isolated from a heterogeneous population of third instar larvae, while here we have selected late stage wandering third instar larvae. Also, in our earlier studies the 0.62 kb *Kpn*I–*Eco*RV probe was used (Eldon *et al.*, 1994); we have noticed that this probe preferentially recognizes the 5.6 kb transcript. Finally, the 2.3 kb transcript may be under tighter temporal or hormonal control than the more ubiquitous 5.6 kb transcript.

Several lines of evidence suggest that the novel transcripts are not degradation products of the 5.6 kb transcript. Utmost care was taken in the preparation of total RNA from

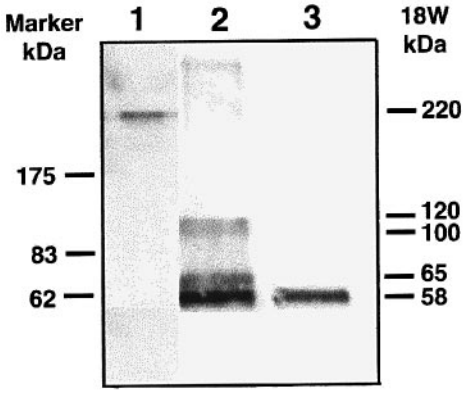


**Fig. 4.** Western analysis of soluble 18W protein. Protein was collected from Oregon$^R$ (lanes 1 and 2) and *18w*$^{7–35}$ homozygous mutant (lane 3) wandering third instar larvae. Lane 1, insoluble protein fraction; lane 2, soluble protein fraction; lane 3, total *18w*$^{7–35}$ protein. The sizes of proteins are indicated on the right and migration of the molecular weight standards are indicated on the left. Proteins were detected using polyclonal anti-18W–XC antibodies raised in rabbits and an HRP-conjugated secondary antibody.

larvae. Live larvae were placed directly into guanidine-containing homogenization solution and all subsequent steps were carried out using standard procedures for working with RNA. The ribosomal bands showed no evidence of degradation (data not shown). In addition, the kinetics of appearance of the four novel transcripts was not consistent with a precursor–product relationship (Figure 3B).

To determine which of the wild-type *18w* transcripts are present in mutants and whether they are similarly induced by infection, wandering third instar Oregon$^R$ and *18w*$^{7–35}$ larvae were subjected to bacterial challenge. RNA was isolated 1 h after infection and wild-type and mutant transcripts were compared (Figure 3C). In Oregon$^R$ larvae the five *18w* transcripts were seen (lane 2), but in *18w*$^{7–35}$ only a 2.3 kb transcript was visible (lane 1). Unlike wild-type larvae, in *18w*$^{7–35}$ mutants the 2.3 kb transcript was not induced and the other transcripts were never seen upon infection with *Escherichia coli*.

The presence of novel transcripts suggested that more than one form of the 18W protein could be present in larvae. Western analysis was performed to determine whether novel forms of the protein were produced. To distinguish between membrane-bound and soluble forms, which might account for the signal detected in the cytoplasm in uninfected larval fat body, mutant and wild-type larvae were fractionated by isotonic lysis to yield soluble and insoluble protein fractions. Figure 4 demonstrates that in addition to one insoluble >200 kDa protein (lane 1) reported in embryos by Eldon *et al.* (1994), four novel soluble proteins were found in wild-type larvae (Figure 4, lane 2). These proteins had apparent molecular masses of 120, 100, 65 and 58 kDa and may account for the signal detected in the cytoplasm of Oregon$^R$ fat body. In *18w*$^{7–35}$ larvae only a 58 kDa protein was produced (Figure 4, lane 3), which may account for the cytoplasmic 18W protein detected in mutant fat body (Figure 2C). The insoluble protein reported here consistently ran above 200 kDa. The Western data shown in Figure 4A were obtained using a polyclonal antibody raised against an intracellular domain of the protein (Materials and methods), but all five protein forms were also detected with an antibody raised against a portion of the extracellular domain of the 18W protein (data not shown). The four novel proteins are not found in embryos, the only stage at which Western detection was previously carried out (Eldon *et al.*, 1994). It had previously been reported that the large insoluble protein was ~170 kDa. The size discrepancy between earlier reports and this report may be due to differing gel conditions, different markers or both.

### *Viability of 18-wheeler mutants following bacterial challenge*

*18w* is expressed in immune responsive tissues and is itself induced following bacterial challenge, but a more direct test for a role in the immune response would be to determine whether the ability to combat infection was compromised in mutant larvae. The *18w*$^{7–35}$ mutant is not null, but mutant larvae produce only a single transcript and protein, which are not induced following infection. This suggests that mutants may show a reduced ability to combat infection, which would be reflected in decreased viability in response to bacterial challenge. To control for non-specific effects due to genetic background, a homozygous viable precise excision of the *PZ* element in l(2)00053 (*18w*$^{1–12}$) was tested in addition to *18w*$^{7–35}$ homozygotes, *18w*$^{7–35}$ heterozygotes and Oregon$^R$. All four lines of larvae were infected with a concentrated solution of *E.coli* or *Enterobacter cloacae* and observed for 24 h. The onset of pupation prevented longer survival times from being tested. Approximately 90% of the Oregon$^R$, *18w*$^{1–12}$ and heterozygous *18w*$^{7–35}$ mutants survived, while only 56% of the homozygous *18w*$^{7–35}$ larvae were still alive after 24 h (Figure 5). The fact that the homozygous *18w*$^{1–12}$ larvae survived bacterial challenge demonstrates that reduced viability was not due to genetic background. Similarly, the survival of 100% of both wild-type and *18w*$^{7–35}$ homozygous mutant larvae when wounded with a sterile pyrogen-free needle (Figure 5) demonstrates that the high mortality of mutants was a specific result of infection and not a result of decreased overall viability or inability to survive wounding. We conclude that the reduced viability observed in homozygous *18w*$^{7–35}$ larvae relative to wild-type larvae is due to a faulty response to bacterial challenge by larvae homozygous for the *18w*$^{7–35}$ mutation.

### *Dif translocation*

To investigate the mechanism by which the *18w* mutation caused increased mortality and to determine whether *18w* participated in a Rel signaling pathway, we quantified nuclear and cytoplasmic levels of Dif and Dorsal protein following infection in wild-type and mutant larvae. Initial experiments employed antibodies raised against Dif (see Materials and methods) to compare immunofluorescence intensity of nuclei versus cytoplasm in dissected fat body. While those assays showed a striking qualitative defect in nuclear translocation, the difference was difficult to quantify.

To quantify Dif localization, nuclear and cytoplasmic protein extracts were prepared from infected and uninfected larvae and subjected to Western blotting and immune detection (Figure 6A, top panel). Two bands were detected,

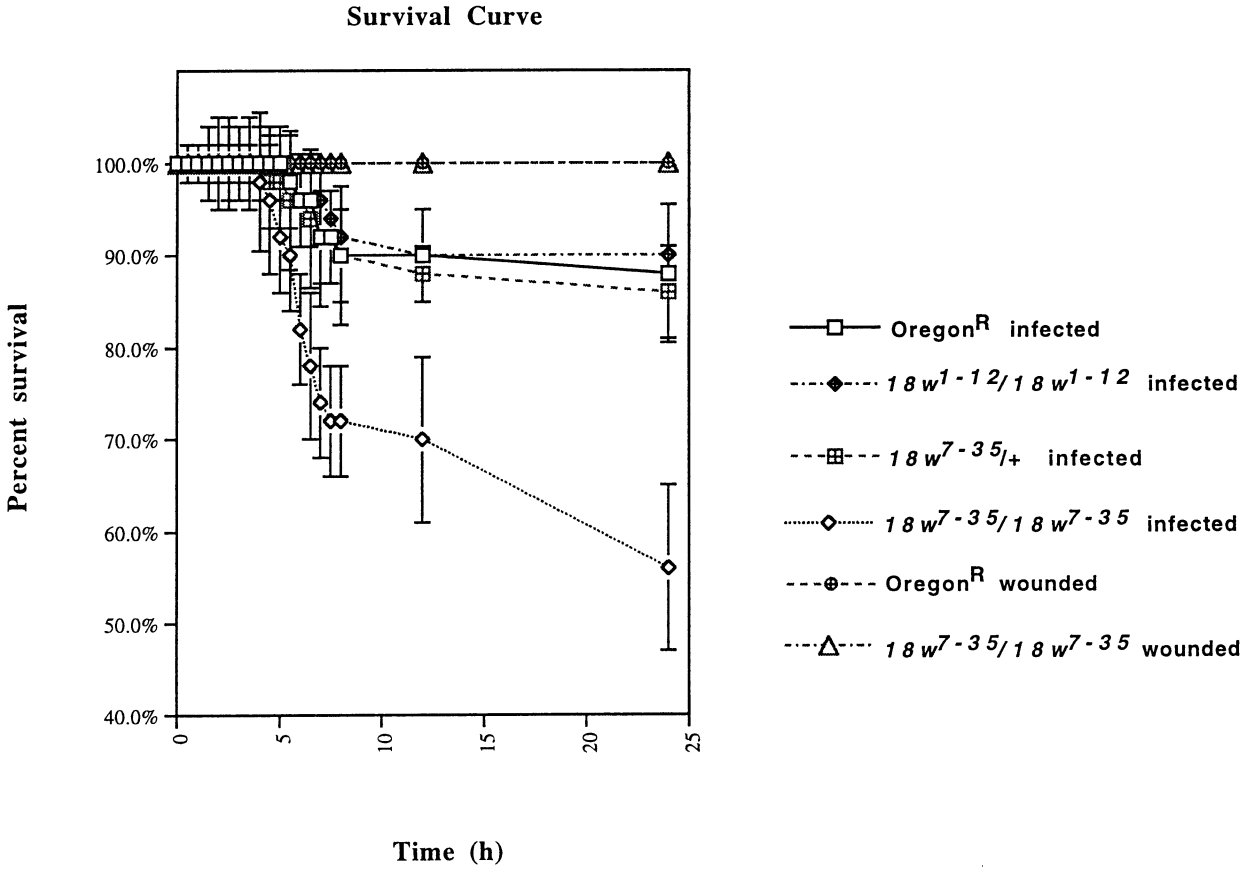


**Fig. 5.** Survival of third instar larvae after wounding or inoculation with *E.coli* bacteria. The survival rates of Oregon$^R$, homozygous *18w*$^{7–35}$ mutants, heterozygous *18w*$^{7–35}$ mutants and *18w*$^{1–12}$ revertants are shown. Confidence levels ($P < 0.005$) are based on five repetitions using 10 larvae for each time point. Larvae were either wounded with a sterile glass needle dipped into sterile *Drosophila* Ringer solution or infected with *E.coli* at the $LD_{10}$ for wild-type larvae. Following treatment the larvae were placed on sterile grape juice plates and observed for 24 h.

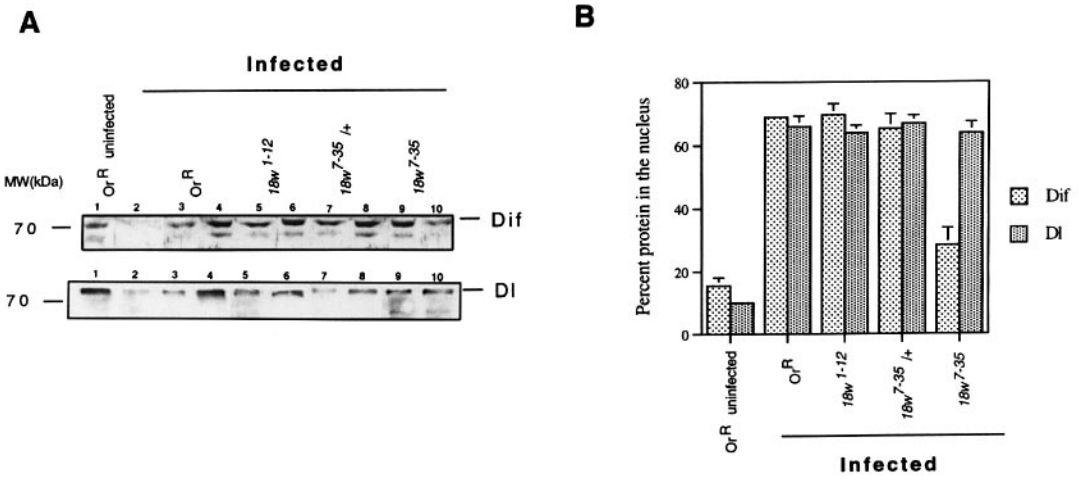


**Fig. 6.** Western analysis of Dif protein translocation in infected third instar larvae. (**A**) Western analysis of Dif translocation in Oregon$^R$, *18w*$^{1–12}$, *18w*$^{7–35}$/T(2;3)CyO;TM6b,Tb and homozygous *18w*$^{7–35}$ wandering third instar larvae infected with log phase *E.coli*. Cytosolic and nuclear proteins were collected prior to infection (lanes 1 and 2) or 90 min after infection (lanes 3–10). (Top) Anti-Dif antibody and a HRP-conjugated secondary antibody were used to detect Dif protein. (Bottom) Anti-Dorsal antibody and a HRP-conjugated secondary antibody were used to detect Dorsal protein. Lanes 1, 3, 5, 7 and 9 are cytoplasmic proteins and lanes 2, 4, 6, 8 and 10 are nuclear proteins. (**B**) Levels of Dif (top band) and Dorsal were measured densitometrically using an Ultrascan XL laser densitometer. Data are presented as the percentage of total protein found in the nucleus [nuclear/(nuclear + cytoplasmic)×100]. Stippled bars represent Dif protein profiles and shaded bars represent Dorsal protein profiles. Confidence levels are indicated ($P < 0.005$).

a major band with an expected electrophoretic mobility based on the predicted Dif sequence and a minor band migrating slightly more rapidly. The identity of this lower band is not known, but its behavior mimics that of Dif. The lower panel of Figure 6A represents the same assay carried out using Dorsal antiserum.

The results of six independent repetitions are presented in Figure 6B. The stippled bars on the graph depict that fraction of total cellular Dif protein represented by the major band, and the shaded bars represent that fraction of total cellular Dorsal protein found in the nucleus. In wild-type larvae prior to infection only 15% of cellular Dif protein was nuclear. Similar results were seen in *18w*$^{1–12}$, *18w*$^{7–35}$ heterozygtes and *18w*$^{7–35}$ homozygotes prior to infection (data not shown). In Oregon$^{R}$ larvae 90 min after infection 69% of the Dif protein was found in the nucleus, confirming the results of Ip *et al.* (1993). *18w*$^{1–12}$ control animals and heterozygous *18w*$^{7–35}$ larvae showed nuclear levels of Dif following infection that were not significantly different from wild-type. In homozygous *18w*$^{7–35}$ larvae, however, the nuclear levels of Dif were only slightly elevated following infection: 28% of the cellular Dif protein was found in the nucleus and 72% remained cytoplasmic (Figure 6B). In contrast to Dif, the nuclear translocation of Dorsal following infection is not significantly affected in *18w*$^{7–35}$ larvae. Approximately 10% of the total cellular Dorsal protein is nuclear prior to infection in wild-type larvae, but following infection 65–70% of total cellular Dorsal protein is nuclear in all cases.

Several points emerge from these experiments. First, Dif protein is present at low levels in the nucleus of uninfected larvae, consistent with previous results (Ip *et al.*, 1993). Second, the level of Dif protein in the nucleus increases dramatically following infection in wild-type, but not *18w* mutant larvae. Finally, the nuclear import of Dorsal is unaffected in *18w*$^{7–35}$ larvae. Thus at least one component of the immune response, the nuclear import of the transcription factor Dif, is significantly reduced in *18w* mutants. Dif has been shown to be active in inducing *cecropin* synthesis in cultured cells (Petersen *et al.*, 1995), but results obtained with *Toll*$^{D}$ mutants suggest that nuclear Dif localization alone is insufficient to induce antimicrobial peptide synthesis (Lemaitre *et al.*, 1996). While Dorsal is active in inducing *cecropin* and *diptericin* synthesis in a cell culture assay (Gross *et al.*, 1996; Petersen *et al.*, 1995), its role in the immune response is less clear. The promoter regions of several antimicrobial genes contain a complex arrangement of transcription factor binding sites (reviewed by Hultmark, 1993), suggesting that more than a single factor is required to initiate antimicrobial peptide synthesis. It is worth noting in this context that we have not yet determined the role *18w* plays in the putative processing and nuclear import of Relish, the third Rel protein identified in flies.

### *Antimicrobial peptide synthesis*

To determine whether the failure of Dif protein to become localized in the nucleus in an *18w* mutant and the increased sensitivity of *18w* mutants to bacterial infection are correlated with reduced transcription of antimicrobial peptide genes we analyzed *cecropin*, *diptericin* and *attacin* transcript levels following infection in wild-type and mutant larvae. Total RNA was isolated 3.5 h after infection of larvae with *E.coli* (Figure 7). Figure 7A shows a Northern blot of total RNA isolated from control and mutant larvae. At least three independent blots were scanned densitometrically to generate the values plotted in Figure 7B and C. The graphs allow direct comparison of levels of *cecropin*, *diptericin* and *attacin* transcript in wild-type and mutant larvae prior to infection (Figure 7B) and 3.5 h after infection (Figure 7C). For each experiment the level of transcript accumulating in wild-type larvae after infection was assigned the value of 100% induction. The amount of transcript accumulating in mutant larvae is expressed as a percentage of the wild-type level of induction, after correcting for differences in the amount of total RNA present, determined by the *rp49* signal.

Variable levels of *diptericin* transcript were detectable in wild-type larvae prior to infection, but consistently higher levels were detected in mutant larvae prior to infection. High background expression of *diptericin* prior to infection has been seen previously (Reichhart *et al.*, 1992). The slight amount of *cecropin* transcript detected in uninfected *18w* mutant larvae is too low to register on the graph in Figure 7B. *attacin* transcript was undetectable prior to infection. The effect of the *18w* mutation on *cecropin* and *attacin* transcription following infection was dramatic. *cecropin* and *attacin* transcripts reach only 35 and 5% of wild-type levels respectively. This contrasted with the insignificant effect observed for *diptericin*, which reached 88% of wild-type transcript levels following infection.

These results suggest that *18w* mutants may be more susceptible to bacterial infection because they are unable to synthesize normal levels of antibacterial peptides. They also constitute striking evidence of differential regulation among the antibacterial peptide genes. In the *18w*$^{7–35}$ mutant *attacin* is reduced by 95%, *cecropin* synthesis is reduced by 65% and *diptericin* synthesis is reproducibly reduced by a slight 12%. This strengthens the proposition that components of the *Drosophila* immune response are independently regulated (Lemaitre *et al.*, 1995a, 1996). Our model, presented in Figure 8, incorporates our data into the immune paradigm (Hultmark, 1994; Lemaitre *et al.*, 1996) by hypothesizing at least two independent pathways differentially regulating the three antibacterial peptide genes whose expression we have analyzed.

## Discussion

We have demonstrated that *18w* plays a critical role in mediating the humoral immune response. *18w* is expressed in tissues important in host defense. Relative to wild-type larvae, *18w* mutant wandering third instar larvae show decreased viability in the face of bacterial challenge. They also show reduced nuclear import of Dif, but not of Dorsal, and reduced levels of antimicrobial transcript accumulation following infection. Two unexpected findings emerged as well. First, novel *18w* transcripts are induced following infection accompanied by the presence of smaller 18W proteins in wandering third instar larvae. Second, an *18w* mutant has different effects on *attacin*, *cecropin* and *diptericin* synthesis, suggesting that the antimicrobial genes are independently and differentially regulated.

Previous sequence analysis suggested that the *18w* gene encoded a receptor. Our data show that 18W protein is found primarily at the cell periphery, consistent with membrane localization. It is worth noting that no cell polarity is revealed by 18w localization; all surfaces of the cell show protein accumulation. In fat body 18W

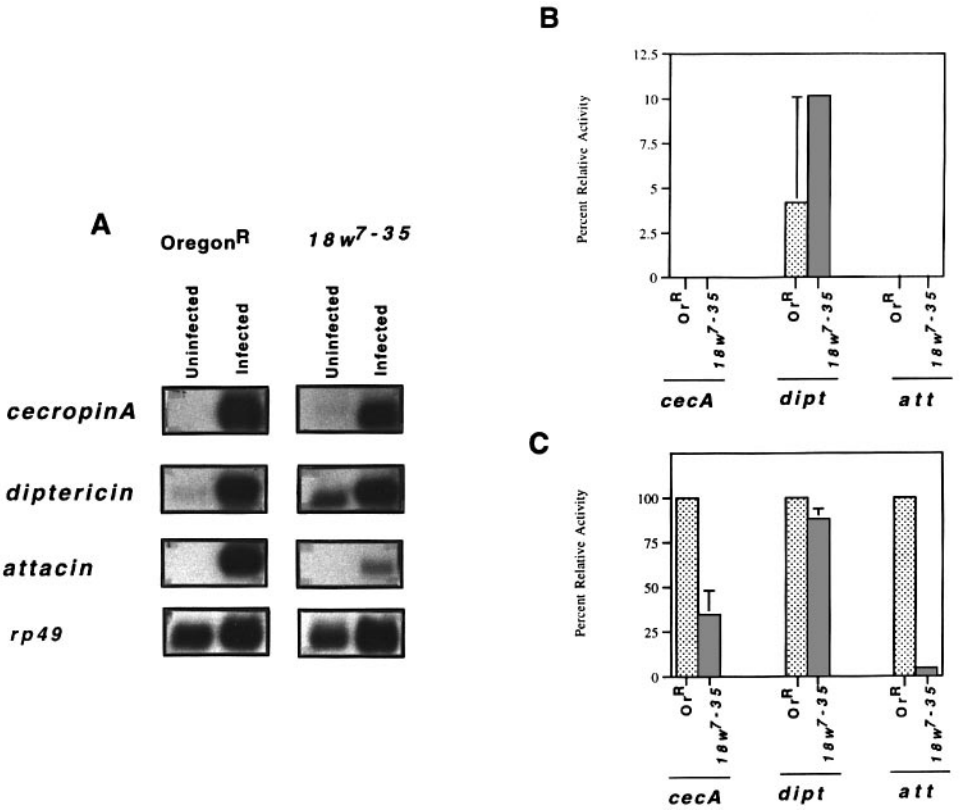


**Fig. 7.** Northern analysis of antibacterial genes in Oregon$^R$ and *18w*$^{7–35}$ mutant third instar larvae. (**A**) Northern analysis of antibacterial gene transcript accumulation in Oregon$^R$ and *18w*$^{7–35}$ larvae infected with log phase *E.coli* at the wild-type $LD_{40}$. Total RNA was collected from uninfected larvae and larvae 3.5 h after infection. The blots were hybridized with the following random primed cDNA probes: *cecropin A1*, *diptericin*, *attacin* and *rp49*. (**B** and **C**) The signals of three Northern blots similar to those represented in (A) were measured densitometrically using an Ultrascan XL laser densitometer and corrected for RNA loading using the *rp49* signal. The levels of expression in infected Oregon$^R$ larvae were standardized as 100% and the levels of expression in uninfected and*18w*$^{7–35}$ larvae are expressed as percent relative accumulation. (B) Induction profile of uninfected Oregon$^R$ and *18w*$^{7–35}$ wandering third instar larvae. (C) Induction profile of infected Oregon$^R$ and *18w*$^{7–35}$ wandering third instar larvae. The transcript induction profiles are shown with their respective confidence level: *cecropin A1* ($P < 0.005$); *diptericin* ($P < 0.05$); *attacin* ($P < 0.001$).

protein can also be detected in cytoplasmic vesicles found just under the plasma membrane (M.Williams, unpublished observation). At this time we are unable to determine whether these vesicles are the result of endocytic or exocytic events. While we have yet to identify an extracellular ligand or downstream intracellular signaling proteins, the failure of Dif to be appropriately imported into the nucleus, the effects on cecropin and attacin transcript accumulation and the increased lethality observed upon bacterial challenge all suggest that mutations in *18w* disrupt a signaling pathway.

We do not rule out a role for *18w* in cell adhesion as well, however, since mutant fat body is not as cohesive as wild-type and is easily disrupted (A.Rodriguez and M.Williams, unpublished observations). These two functions are by no means mutually exclusive. The extracellular portion of the protein contains two domains of leucine-rich repeats (LRR). Each LRR domain is flanked by cysteine-rich N- and C-terminal sequences common to LRR-containing glycoproteins (LRGs) (Chiang and Beachy, 1994; Eldon *et al.*, 1994). LRGs, a family that includes *Drosophila* Slit, Connectin and Chaoptin and mammalian glycoproteins Ib and gpX, are known to be involved in protein–protein interactions, which include cell–cell/cell–matrix adhesion as well as ligand binding. Glycoprotein Ib mediates platelet adhesion and binds both α-thrombin and von Willebrand factor (Lopez *et al.*, 1987; Roth, 1992). Toll is postulated to act as a receptor in early embryogenesis, then as an adhesion or cell recognition molecule during later developmental processes (Gerttula *et al.*, 1988; Halfon *et al.*, 1995).

18W protein accumulates in several tissues in wandering third instar larvae, including the fat body, the pro-hemocytes in the lymph glands and the garland cells. All of these cells are known to synthesize and secrete substances into the hemolymph. The fat body is a major site of antimicrobial peptide production. Since 18W protein is seen in vesicles in the fat body, it may be a major site of synthesis for the novel soluble forms of 18W, a possibility we are currently testing.

Pro-hemocytes are precursor cells found in the lymph gland that give rise to circulating hemocytes. Plasmatocytes, one class of hemocyte, are the migratory phagocytic cells of the *Drosophila* immune response that secrete antimicrobial peptides, while crystal cells, another class of hemocyte, secrete components of the phenoloxidase cascade involved in encapsulation and melanization. 18W expression by pro-hemocytes is consistent with a role for 18W in immune signaling and possibly hemocyte maturation. We are currently investigating whether 18W protein is present in mature circulating hemocytes. Garland cell expression of 18W is also intriguing, since garland

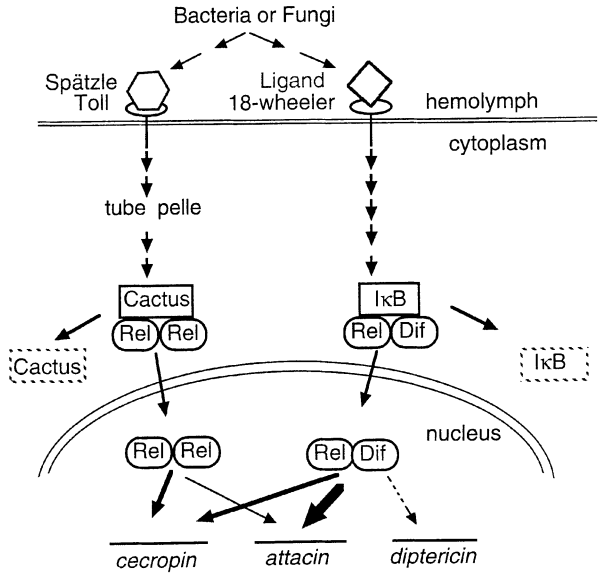


**Fig. 8.** Model for the control of antimicrobial genes expression in *Drosophila* wandering third instar larvae. Lemaitre *et al.* (1996) have shown that the Toll signaling pathway is necessary for full induction of *drosomycin*, *cecropin*, *attacin* and *defensin*. Our research has shown that there is further complexity in the regulation of the *cecropin*, *diptericin* and *attacin* genes. *cecropin* seems to require both the Toll–Rel and the 18W–Dif pathways for full activation. *diptercin* is not affected by mutations in the Toll pathway and its expression is not significantly reduced in an *18w* mutant. *attacin* is also not significantly reduced by mutations in the Toll pathway, but is reduced by 95% in an *18w* mutant. Multiple arrows indicate unknowns. For example, it is not clear how Spätzle is processed in immunity or what cytoplasmic proteins interact directly with the cytoplasmic domain of Toll or with Cactus. The identity of the Rel proteins bound by Cactus is not clear nor is it clear whether homo- or heterodimers (or both) of Rel proteins are regulated by Cactus. Even more questions remain about components of the 18W pathway, including the identities of the ligand upstream and the IκB protein downstream. What is clear is that the two signaling pathways mediate different effects on antibacterial peptide synthesis in response to microbial infection.

cells have been shown to absorb toxins from the hemolymph (Rizki, 1978). They are also thought to be major sites of synthesis for peptides and proteins that are secreted into the hemolymph in *Manduca sexta* (Dickinson *et al.*, 1988).

The viability of *18w* mutants is severely compromised in the face of bacterial challenge. We have ruled out the possibility that *18w* mutants are less fit and more sensitive to injury by demonstrating that they survive sterile wounding as well as wild-type wandering third instar larvae. Under conditions in which 90% of infected wild-type wandering third instar larvae survived only 56% of the mutants survived. This differs from adult *Toll* mutants, where no significant difference in viability is observed between mutants and Oregon$^R$ following bacterial infection (Lemaitre *et al.*, 1996). When *immune deficiency* (*imd*) adults are infected with levels of bacteria that allow 90% wild-type survival, only 10% of the mutants survive (Lemaitre *et al.*, 1995a). *imd* mutants induce only *drosomycin*, an antifungal peptide, upon microbial challenge and are unable to induce antibacterial peptide transcription (Lemaitre *et al.*, 1995a). While care must be taken in comparing the immune response in adults with that in larvae, it is possible that the observed reduction in antibacterial protein induction in *18w* mutants accounts for the full extent of reduced viability. We have not ruled out the possibility that *18w* plays other essential roles in the immune response in addition to its role in the antimicrobial peptide signaling cascade.

One aspect of the antimicrobial peptide signaling cascade that is affected in *18w* mutants is nuclear localization of Dif. However, it has been well documented that nuclear localization of Dif is not sufficient to initiate transcription of antimicrobial genes. Corbo and Levine (1996) report that Dif appears to be properly translocated in adults mutant for the immunodeficiency locus at 55C-F (called *imd* by Lemaitre *et al.*, 1995a), yet *imd* flies are unable to produce antibacterial peptides (Lemaitre *et al.*, 1995a). Dominant alleles of *Toll* (e.g. *Toll*$^{10b}$) cause accumulation of Dif in the nucleus, but this localization is not accompanied by antimicrobial peptide gene transcription (Ip *et al.*, 1993). The role of Dorsal in regulating antimicrobial synthesis is also unclear. Lemaitre *et al.* (1995b) demonstrated that mutations in which Dorsal is constitutively nuclear, such as *cactus*, do not cause induction of *diptericin* expression. In fact, in the absence of Dorsal protein, flies activate antimicrobial peptide synthesis normally following infection (Lemaitre *et al.*, 1996). Such analyses have not yet been carried out to study the role of Relish in larvae or adults. Thus, while all three Rel proteins have been shown to induce cecropin synthesis independently in transfection studies of cultured cells (Engström *et al.*, 1993; Petersen *et al.*, 1995; Dushay *et al.*, 1996), their roles and relative contributions to Rel dimer formation *in vivo* are less clear. Furthermore, because the nuclear translocation of Rel proteins is inextricably linked to degradation of IκB proteins (Belvin *et al.*, 1995), it will be informative to determine the effect of *18w* mutants on the stability IκBs, such as Cactus and possibly Relish.

The reduced ability of Dif to translocate to the nucleus upon infection in *18w* mutants may provide a useful marker for loss of 18W signaling activity. This reduction correlates with the reduced antibacterial peptide synthesis seen in *18w* mutants, though it may not represent the only effect off *18w* on Rel protein translocation. While we have shown that Dorsal translocation to the nucleus is unaffected in *18w* mutants, we know nothing yet about its effects on Relish.

It is not yet clear whether the differences we observe in antimicrobial transcript accumulation are due to differences between the Toll and 18W signaling pathways or to differences between the immune responses of adults and larvae, but it is clear that *18w* plays an essential role in the response of wandering third instar larvae to bacterial challenge. Unlike adults homozygous for the *imd* mutation, which show nearly complete loss of *cecropin*, *attacin* and *diptericin* inducibility (Lemaitre *et al.*, 1995a), larvae homozygous for the *18w*$^{7–35}$ mutation show differential effects on antimicrobial peptide synthesis. There is a 95% reduction in *attacin* induction and a 65% reduction in *cecropin* induction. The subtle but consistent 12% reduction in *ditericin* induction is not significant. These effects differ from those observed in adult *Toll* mutants, which show reduction by half of *cecropin* and *attacin* and near normal levels of *diptericin* induction (Lemaitre *et al.*, 1996). The most striking difference between the effects of *18w* and *Toll* are on *attacin* induction, though *drosomycin*, *drosocin* and *defensin* have yet to be tested in *18w* mutants.

We have incorporated our results with those of other laboratories in constructing Figure 8. We have drawn *18w* and *Toll* in separate parallel pathways, since their effects on antimicrobial gene expression and Rel nuclear localization are different. Lemaitre *et al.* (1996) have shown that *spätzle*, *Toll*, *pelle* and *tube* show similar effects on *cecropin* gene expression, suggesting they act in the same pathway. However, the effects of *spätzle* and *Toll* mutations on *defensin* synthesis are not identical, suggesting perhaps that another ligand is responsible for *defensin* expression.

We have placed Dif downstream of 18W, though much remains to be learned about control of the nuclear import of Rel proteins. We leave open the possibility that Rel factors in *Drosophila* immunity may function as heterodimers. The boldness of the arrows indicates the relative strength of the effects of each pathway on the synthesis of antimicrobial transcripts. Furthermore, because 18W and Toll do not fully control expression of the known *Drosophila* antibacterial peptides, let alone those that certainly remain to be identified, it is clear that other receptors and pathways remain to be identified as well.

Lastly, the fact that novel forms of the *18w* transcript are induced rapidly following infection raises several fascinating questions about the role of *18w* in the immune response specifically and regulation of the immune response in general. Some of these transcripts may encode secreted soluble receptors, since all four 18W soluble proteins contain LRRs and the IL-lR homology domain and 18W proteins are detected in vesicles in the fat body prior to infection. Soluble receptors are important modulators of innate immunity in vertebrates. Mammalian CD14 is a LRR-containing glycoprotein that exists in both a soluble form and a GPI-linked membrane bound form found on the surface of monocytes and macrophages (Haziot *et al.*, 1996). Soluble forms of CD14 have been shown to be important intermediates in lipopolysaccharide-induced cell activation (reviewed by Ulevitch and Tobias, 1995), while soluble IL1-R are the most effective means of decreasing the inflammatory effects of IL1 (reviewed by Dinarello and Wolff, 1993). We do not yet know whether soluble forms of 18W have inhibitory or stimulatory effects on host defense.

The conservation of aspects of this pathway is remarkable. Resistance to infection in plants has been shown in some cases to involve LRR-containing proteins. The tomato *N* gene encodes a cytoplasmic protein containing LRRs and an IL-1R homology domain and the *N* protein is necessary in a signaling cascade leading to tobacco mosaic virus resistance (Whitham *et al.*, 1994). This remarkable conservation of pathways in the immune responses of a variety of organisms as well as in *Drosophila* development has been discussed extensively (see for example Hultmark, 1993; Wasserman, 1993; Belvin and Anderson, 1996) and testifies to the efficiency of the IL-1R–Rel pathway in effecting rapid and specific changes in transcriptional activity.

## Materials and methods

### *Drosophila stocks*

All *Drosophila* stocks were raised at 25°C on standard cornmeal/brewer's yeast/molasses/agar food. The enhancer detector strain l(2)00053 (Karpen and Spradling, 1992) has a *PZ* (Mlodzik and Hiromi, 1992) P element ~400 bp 5′ of the *18w* open reading frame (ORF). It has been shown to express β-galactosidase in an *18w* pattern during embryogenesis (Eldon *et al.*, 1994). $18w^{7-35}$ and $18w^{1-12}$ are P element excision alleles, previously described by Eldon *et al.* (1994). $18w^{7-35}$ has a 2.2 kb deletion extending from the site of P element insertion into the *18w* ORF, $18w^{1-12}$ is a wild-type reverent. Heterozygous $18w^{7-35}$ were kept over the T(2;3)CyO;TM6B,Tb balancer.

### *Detection of β-galactosidase activity*

The procedure for β-galactosidase staining is a modification of *Drosophila* Laboratory Manual Protocol 77 (Ashburner, 1989). Wandering third instar larvae were dissected in phosphate-buffered saline (PBS), pH 7.0, fixed for 15 min in a 1% glutaraldehyde solution in PBS and washed three times for 5 min in PBX (PBS plus 0.3% Triton X-100). Fixed samples were incubated for 4 h in a 0.2% X-gal staining solution (10 mM ferrocyaninde, 10 mM ferricyanide, 0.5 μM $MgCl_2$ solution in PBX). After staining the samples were washed three times for 5 min in PBS and mounted in 70% glycerol in PBS. All photographs were taken using a Nikon Optiphot-2.

### *Tissue in situ hybridization*

Tissue *in situ* hybridization to larval tissues was carried out according to the procedure of Ephrussi *et al.* (1991) for adult ovaries, with the following modifications. Third instar larval fat body and additional tissues were treated with 50 μg/ml proteinase K for 15 min and the second paraformaldehyde fixation was for 30 min. The probe was a 3.5 kb cDNA fragment, encoding approximately amino acids 145–1230 of the *18w* ORF, labeled with digoxygenin and detected using a non-radioactive DNA labeling and detection kit (Boehringer Mannheim).

### *Immunohistochemistry on wandering third instar larvae*

*18w antibody production.* A 356 bp *Nae*I–*Kpn*I restriction fragment encoding amino acids 1065–1159 of the intracellular domain of the *18w* ORF was inserted into *Stu*I-digested plasmid pMAL-c (New England Biolabs) to create a maltose binding protein–18W–IC fusion protein. Bacterially expressed protein was affinity purified and injected i.m. into white leghorn laying hens. IgY was purified by PEG precipitation from the egg yolk produced by immune hens (Polson *et al.*, 1980) and affinity purified.

A 1.1 kb *Eco*RV restriction fragment encoding amino acids 576–948 of the extracellular domain of the *18w* ORF was inserted into *Stu*I-digested plasmid pMAL-c (New England Biolabs) to create a maltose binding protein–18W–XC fusion protein. Insoluble bacterially expressed protein was eluted from preparative denaturing SDS–polyacrylamide gels and injected s.c. into New Zealand White rabbits. IgG was purified from immune serum using Affi-Gel Blue columns (BioRad) and stripped of non-specific reactivity by incubation with Amino-link resin (Pierce) coupled to the soluble fraction of the bacterial extract.

*Whole tissue.* Wandering third instar larvae were dissected in PBS, pH 7.0, fixed for 15 min in 4% formaldehyde solution in PBS and washed three times for 15 min in PBS. Fixed samples were preincubated for 1 h in blocking solution [2% bovine serum albumin (Boehringer Mannheim), 2% normal rabbit serum and 0.1% Tween 20 solution in PBS]. The samples were incubated in primary antibody (1:10 anti-18W) in blocking solution overnight at 4°C on a shaker. The samples were washed four times for 5 min in blocking solution and then incubated for 1 h at room temperature in diluted rabbit anti-chicken horseradish peroxidase (HRP)-conjugated antibody (1:250 dilution; Pierce, Rockford, IL). The secondary antibody had been pre-absorbed against several fixed wandering third instar larvae overnight to reduce background signal. Samples were then washed four times for 15 min in PBT (PBS and 0.1% Tween 20). The HRP reaction was performed using a 0.5 μg/ml solution of 3,3′-diaminobenzidine (DAB) in PBS and 0.01% hydrogen peroxide for 5–10 min, resulting in an orange/brown stain. The samples were rinsed once in double distilled $H_2O$ and once in PBS to stop the reaction. Samples were then mounted in 70% glycerol in PBS and photographed using a Nikon Optiphot-2.

### *Northern blot analysis*

Total RNA extraction was performed using the Ultraspec RNA Isolation System (Biotecx, Houston, TX). Total RNA aliquots (10 μg) were separated on a 1% agarose–7% formaldehyde gel, stained with ethidium bromide and transferred overnight to nylon membrane (Micron Separations Inc., Westboro, MA). The membranes were cross-linked using a Stratalinker. The following random primed labeled cDNA probes were utilized to detect *18w*, *cecA*, *dipt* and *att* RNAs: a cDNA probe

corresponding to a 1.0 kb *Eco*RV fragment of the *18w* coding region; a 300 bp *Sal*I–*Kpn*I fragment of the *18w* coding region (Figure 3D; Eldon *et al.*, 1994); a *cecA1* cDNA probe (Kylsten *et al.*, 1990); a *dipt* cDNA (Wicker *et al.*, 1990); an *att* cDNA (Åsling *et al.*, 1995). Hybridization of a *rp49* oligonucleotide probe (O'Connell and Rosbash, 1984) was used to standardize RNA loading. Northern blot analyses were repeated at least three times. The relative extent of hybridization was evaluated by densitometry (Ultrascan XL laser densitometer; Pharmacia LKB, NJ).

#### *Viability experiments*

Bacterial challenge was performed by injuring adults with a pyrogen-free sterile glass needle previously dipped into a concentrated bacterial culture of *E.coli* or *E.cloacae* washed in sterile *Drosophila* Ringer. Wounding experiments were performed by injuring larvae with a pyrogen-free sterile glass needle previously dipped into sterile *Drosophila* Ringer. Groups of 10 wandering third instar larvae were inoculated, transferred to sterile grape juice plates and observed over the next 24 h at the indicated times. Viability experiments were repeated at least five times.

#### *Isolation and Western analysis of cytosolic and nuclear Dif and Dorsal*

Protein isolation was carried out as described by Olnes and Kurl (1994). Oregon$^R$, *18w*$^{7–35}$, *18w*$^{7–35}$/+ and *18w*$^{1–12}$ wandering third instar larvae were collected and washed with PBS, pH 7.0, before being homogenized in 100 µl buffer 1 [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (v/v) aprotinin solution in double distilled $H_2O$]. The homogenate was then incubated on ice for 15 min before being lysed by addition of 0.6% (v/v) Nonidet P-40. The nuclei were pelleted by centrifugation at 2000 *g* for 5 min in a microcentrifuge at 4°C. The supernatant was placed into a new tube and stored at –80°C. The nuclear pellet was resuspended in 50 µl ice-cold buffer 2 [20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1% (v/v) aprotinin and 10% (v/v) glycerol] and shaken at 4°C for 30 min to lyse the nuclei. The nuclear lysate was microcentrifuged at 12 000 *g* for 10 min and the supernatant was recovered to a new tube prior to storage at –80°C. Protein concentrations were quantified using the BioRad DC Protein Assay kit (Hercules, CA). Protein fractions (10 µg) were diluted with sample buffer (1× USB = 125 mM Tris, pH 6.8, 2% SDS, 5% glycerol, 1% β-mercaptoethanol, 6 M urea, 0.003% bromophenol blue), heated at 65°C for 10 min, then centrifuged at 12 000 *g* at room temperature for 30 min. Samples were electrophoresed on a 4% polyacrylamide stacking gel, 12% resolving gel with appropriate size markers (Gibco BRL) until the dye front reached the bottom of the gel. Gels were washed three times for 10 min with transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol), then transferred to a PVDF membrane (Hybond, Amersham). Membranes were blocked for 1 h at room temperature in Western blocking solution (1× TBS with 5% non-fat dry milk, 0.1% Tween-20). The rat anti-Dif antibodies, raised against almost the entire Dif protein (Ip *et al.*, 1993), were diluted 1:1500 in blocking solution and incubated with the membranes overnight at 4°C. Rabbit anti-Dorsal antibodies (Gillespie and Wasserman, 1994) were diluted 1:500 and incubated in the same manner. Membranes were washed three times for 10 min each in blocking solution. HRP-conjugated rabbit anti-rat antiserum (Pierce, Rockford, IL) was diluted 1:10 000 in blocking solution for anti-Dif blots and HRP-conjugated goat anti-rabbit antiserum (Pierce) was diluted 1:10 000 in blocking solution for anti-Dorsal blots. Secondary antibodies were incubated with the membranes for 1 h. Membranes were washed three times for 10 min in blocking solution and then five times for 5 s in TBST (10 mM Tris, pH 8.0, 100 mM sodium chloride and 0.1% Tween 20). Finally, blots were incubated with ECL Western blotting detection reagent (Amersham) for 1 min, then wrapped and exposed to X-ray film (Fuji) for 5–120 s. Western blot analyses were repeated at least six times. The relative amount of antibody binding was evaluated by densitometry (Ultrascan XL laser densitometer; Pharmacia LKB, NJ).

#### *Western blot analysis of 18w*

Oregon$^R$ and *18w*$^{7–35}$ wandering third instar larvae were collected and homogenized in 1× PBS, pH 7.0. The lysate was microcentrifuged at 12 000 *g* for 20 min and the supernatant recovered to a new tube. The concentration was then quantified using the BioRad DC Protein Assay kit (Hercules, CA). Western blot analysis was carried out as above except that rabbit anti-18W antibodies, raised in rabbits against an extracellular domain of the protein, were diluted 1:200 in blocking solution and incubated with the membranes at room temperature for 2 h. Membranes were washed three times for 10 min each in blocking solution. HRP-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL) was diluted 1:10 000 in blocking solution. Blots were incubated with ECL Western blotting detection reagent (Amersham) for 1 min, then wrapped and exposed to X-ray film (Fuji) for 5–120 s. Western blot analyses were repeated at least three times.

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