

# Inside the Immune Response: Isolating Ird20

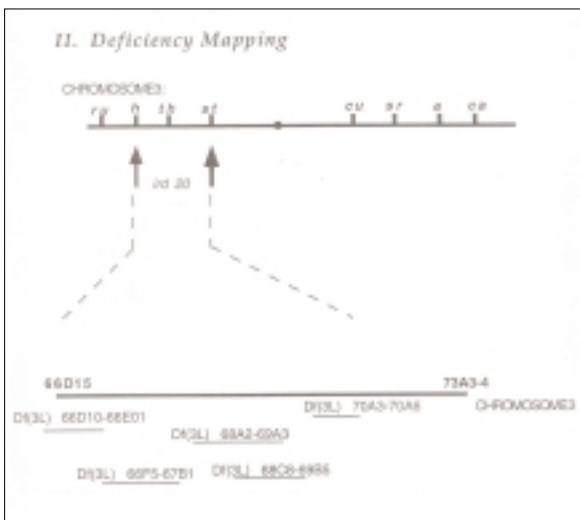
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In both insects and vertebrates, the first rapid response to infection by pathogens is mediated by the innate immune response. This process does not rely on specific recognition of the pathogen, but instead relies on an immediate, general response to a wide range of pathogens without subsequently developing specific, long-term immunity.<sup>1</sup> Cells mediating the innate immune response recognize general features of non-self cells by characteristic chemical differences between microbial cells and host cells. Once the pathogen is recognized, several parallel effector response mechanisms are activated synergistically to resist the invading pathogen. Also, the innate immune response involves the activation of macrophages, which engulf invading pathogens, and the production of antimicrobial peptides, which lyse invading pathogens<sup>2,3</sup> (Figure 1).

Major features of innate immunity appear to be conserved between vertebrates and invertebrates. Both insects and vertebrates contain two major branches of the innate immune response, cellular and humoral. In mammalian innate immune response, the first event is the activation of the cellular response of macrophages, which engulf invading pathogens and secrete cytokines that activate other aspects of the immune response.<sup>4</sup> Analogously, hemocytes (macrophagelike cells) of insects are activated by infection and participate in phagocytosis, encapsulating the invading pathogens<sup>5</sup>. The second event in the mammalian innate immune response involves the produc-

tion of acute-phase proteins by the liver. These proteins are secreted into circulation and are directly involved in killing bacteria. Similarly, the insect fat body (the counterpart to the mammalian liver) produces and secretes a set of antimicrobial peptides in response to infection.<sup>2</sup>

In mammals, NF- $\kappa$ B (a conserved signaling pathway that is used in several, quite different biological processes) has been shown to participate in innate immunity in the response of macrophages and the ensuing production of acute-phase proteins by the liver. Macrophages are activated by bacterial lipopolysaccharide LPS, which triggers the activation of NF- $\kappa$ B and a nuclear factor, NF-IL6. NF- $\kappa$ B and NF-IL6 act in synergy to activate signaling pathways that promote the nuclear translocation of the NF- $\kappa$ B. This translocation, in turn, activates the transcription of other inflammatory



cytokines in blood cells and acute phase proteins in the liver. It has been observed that *Drosophila* antibacterial genes also contain NF- $\kappa$ B- and NF-IL6-binding sites. (Figure 2). Experimental data indicates that, in *Drosophila*, the Toll-Dorsal pathway (which, researchers have found, does have a human homologue) plays a major role in the immune response.<sup>2</sup>

## The Toll-Dorsal Signaling Pathway in *Drosophila* Immune Response

Many signaling pathways are used for related developmental processes in many different kinds of organisms. In addition, a single pathway may be used repeatedly during the life of a single organism to mediate communication between cells in very different biological processes. The Toll-Dorsal pathway is one such pathway. It is repeatedly expressed during *Drosophila* development in a variety of tissues and defines the earliest dorsal-ventral symmetry in the *Drosophila* embryo<sup>2,3</sup> (Figure 2).

Major features of innate immunity appear to be conserved between vertebrates and invertebrates. Recent research indicating that the transcription factors of the Rel/NF- $\kappa$ B family are activated as part of the innate immune response in both mammals and insects has led researchers to compare insect innate immunity and its mammalian counterpart. More remarkably, it has been found that related molecules are applied in plant-disease resistance responses. Several protein families have been identified with homology to components of the Toll signaling pathways.

The Toll-Dorsal pathway in *Drosophila* and the interleukin-1 receptor (IL-1R)-NF- $\kappa$ B signaling pathway in mammals are homologous signal transduction pathways that mediate several different biological processes. Genetic analysis of early dorsal-ventral patterning in *Drosophila* has defined a series of genes that mediate the Toll pathway. The activation of the transmembrane receptor Toll leads to the activation of the cytoplasmic serine/threonine kinase Pelle by the protein tube. Pelle activity controls the degradation of the Cactus protein, which is present in a cytoplasmic complex with the Dorsal protein. Once Cactus is degraded, Dorsal is free to translocate from the cytoplasm into the nucleus, where it regulates the transcription of specific target genes by binding to DNA. The Toll, tube, pelle, cactus, and dorsal genes also appear to be involved in *Drosophila* immune response. Because the IL-1R-NF- $\kappa$ B pathway plays a role in vertebrate innate immunity and because plant homologues

of the Toll pathway appear to play a vital role in plant disease resistance, it is likely that this pathway arose before the divergence of plants and animals as a defense against pathogens<sup>2</sup>.

The Toll-Dorsal pathway was originally found to define early dorsal-ventral polarity in *Drosophila* embryo. The same pathway is activated in the fat body of larvae and adults as a part of the immune response, to activate the production of some of the antimicrobial peptides (Figure 3). Microbial infection triggers the Rel proteins Dorsal and Dif to translocate from the cytoplasm to the nuclei of fat body cells (occurs within 30 minutes after infection). Here, they activate the transcription of genes that play a major role in the immune response. In the early *Drosophila* embryo, the Toll signaling pathway controls the nuclear import of Dorsal in the immune response. However, studies show that the Toll pathway is not required for the import of Dif 3. It has been found that all three known Rel family members, Dorsal (dl), Dif (Dorsal-related immunity factor), and Relish, are activated in response to infection<sup>6,7,8</sup> (see Figure 3).

The Toll pathway plays a very important role in *Drosophila* immunity. Recent experiments have unraveled the mystery concerning the production of the antifungal gene Drosomycin. The Toll pathway is critical in this phase of the immune response. However, uncertainties remain about the role of the Toll pathway in the production of anti-bacterial peptides.

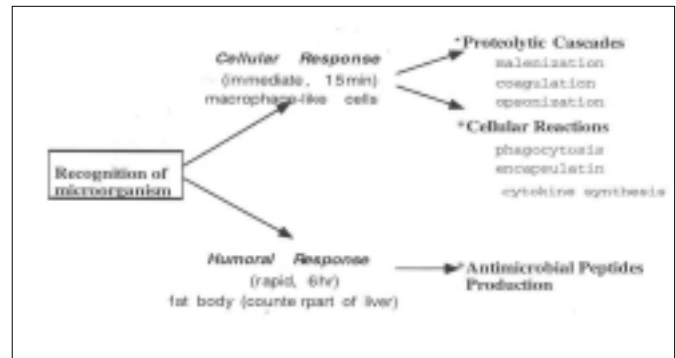


Figure 1

## *Drosophila* Rel Family Proteins

In *Drosophila*, all three known Rel family members (Figure 4) are activated in response to infection. Although the activation of the three *Drosophila* Rel proteins is the best-characterized aspect of the insect immune response, little is known about the signals that activate each Rel protein and the specific targets of the Rel proteins. To date, seven genes encoding antibacterial peptides of *Drosophila* have been cloned. These include four cecropins, diptericin, defensin, and drosocin.<sup>7</sup>

In addition to its function in embryonic development, the transcription factor Dorsal (dl) has been shown to be expressed in larval and

adult fat body where its RNA expression is enhanced upon injury. Injury leads to a rapid nuclear translocation of dorsal from the cytoplasm in fat body cells. Previous studies have shown that the nuclear localization of dorsal is controlled by the Toll signaling pathway. More importantly, studies have shown that injury induced expression of the antibacterial peptide coding gene dipterin can occur in the absence of dorsal. These data underline the complexity of the *Drosophila* immune response<sup>7</sup>.

Dif is a novel Rel-containing gene in *Drosophila* that provides a potential link between the seemingly unrelated processes of dorsal-ventral patterning and immune response. Dif maps close to dorsal (hence the name dorsal-related immunity factor), yet it does not seem to be involved in dorsal-ventral patterning. Rather, Dif mediates an immune response in *Drosophila* larvae. Normally, Dif is localized in the cytoplasm of larval fat body, but, like dorsal, quickly translocates to the nucleus of fat body cells upon

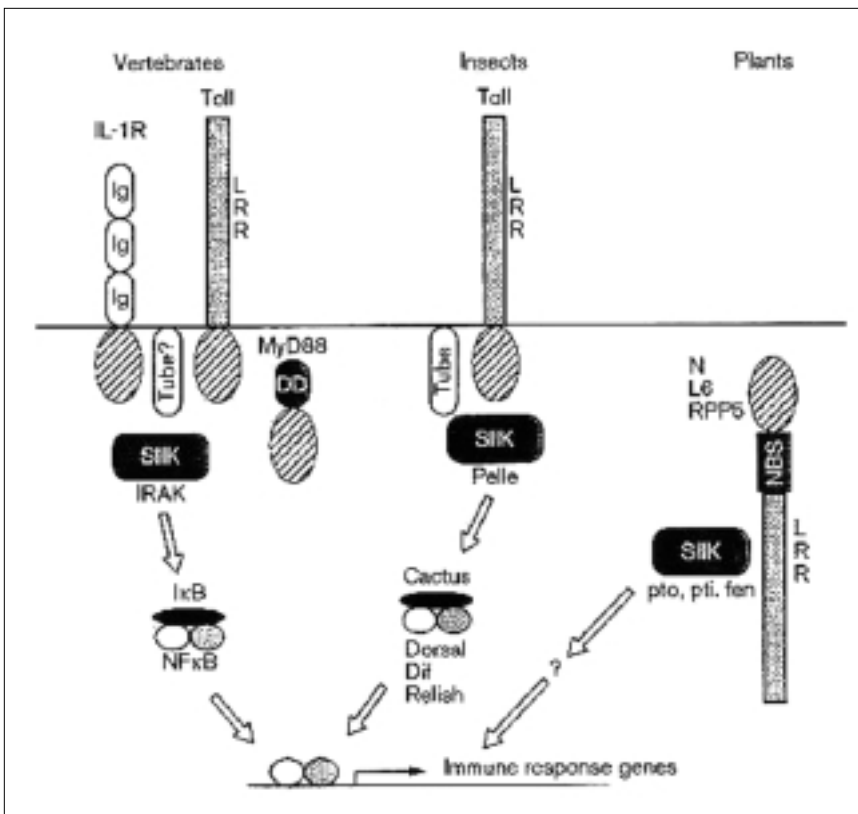
lymph, play a vital role in the insect immune response. Cells of the fat body and components of the hemolymph also provide essential components in the immune response. Plasmatocytes become activated in response to infection, which initiates the phagocytosis of pathogens. In addition, lamellocytes--differentiated, flattened plasmatocytes--encapsulate foreign pathogens.<sup>9</sup> After encapsulation, melanin is deposited around the pathogen. Melanin kills invading microorganisms, but has no effect on host cells. 10 Crystal cells, a second class of blood cells, secrete important components into the hemolymph, including components activating a prophenoloxidase cascade, leading to melanization.<sup>9</sup>

The fat body, like the liver in mammalian systems, is the site of synthesis of proteins involved in killing pathogens. More than fifty antibacterial peptides have been purified from the hemolymph of infected insects.<sup>11</sup> The largest gap in our understanding of the innate immune response remains how antibacterial peptide genes are induced in response to infection.<sup>12</sup> (see Figure 4).

Previous studies have shown that in *Drosophila* all three known Rel family members, are activated in response to infection. The Rel Domain, characteristic of all Rel family proteins, is the DNA-binding portion of these transcription factors. Ankyrin repeats are a specific sequencing characteristic of Relish alone.

In review: It has been found that insect immune response occurs in two phases, analogous to the mammalian initial response to infection. Hemocytes blood cells, activated by infection, phagocytize invading pathogens. Later, fat body cells secrete antimicrobial peptides in response to infection. The Toll-Dorsal pathway has been defined as playing a vital role in *Drosophila* immune response. However, the largest gap in our understanding remains how the antimicrobial peptides are induced. The rapid induction of transcription of genes encoding antimicrobial peptides in the fat body is the best-characterized aspect of the insect immune response. However, little is known about the signals that activate each Rel protein and the specific targets of the Rel protein. Moreover, other signaling pathways activated in the immune response and possible "crosstalk" between different pathways are poorly understood. Research shows that larvae and adults lacking only Toll normally induce the transcription of these peptides in response to infection. Thus, other unknown signaling pathways must also be activated upon infection.

The mutations that have been isolated, *ird 20* being of particular interest, should identify components of these unknown signaling pathways



**Figure 2** bacterial infection or injury. Upon entering the nucleus, evidence suggests that Dif binds to promoter regions of immunity genes, beginning a series of events directly involved with lysing invading microorganisms.<sup>6</sup>

### Insect Immunity

Two classes of blood cells, arising in the

and may also identify other unknown components of the Toll pathway. A specific goal of this study is to explain the functions and regulation of the Rel family proteins in mediating the *Drosophila* immune response. Thus, by manipulating DNA sequences in the third chromosome of the *Drosophila* genome and assessing the absence of larval immune response, it is possible to find the essential components involved in the *Drosophila* immune system upon bacterial infection.

## Experimental Design

### List of Materials

Fly stocks (wild-type/different mutant lines); Fly larvae; Incubator (18°C, 37°C); Pipettes; Centrifuge; Microinjection needle and syringe; PBS phosphate buffered saline; X-gal solution (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside); Glutaraldehyde; 10 mM NaPO<sub>4</sub> buffer (pH 7.2); 150 mM NaCl; 1 mM MgCl<sub>2</sub>; 3.1 mM K<sub>4</sub>[FeII(CN)<sub>6</sub>]; 3.1 mM K<sub>3</sub>[FeIII(CN)<sub>6</sub>]; 0.3% Triton X-100; Bacterial solution (early log phase *E. coli*); Fixative solution; Staining solution; Paraformaldehyde; PBTA solution (1% Triton X-100 and 1% Bovine Serum Albumin in PBS); Rabbit Anti-Dif antibody; Mouse monoclonal anti-Dorsal antibody; Fluorescein isothiocyanate goat-anti-rabbit-IgC serum; Rhodamine-conjugated goat-anti-mouse-IgC serum; and glycerol.

## Procedures

### Diptericin Expression

Since little is known about the specific activation of diptericin, an antibacterial peptide, a systematic genetic analysis has begun, in which the diptericin-lacZ gene is being used as a reporter. Previously, over 40 recessive mutations were isolated on the third chromosome of *Drosophila* for failure to induce diptericin. These genes were named *ird* genes—immune response deficient. These mutations are currently in the process of being isolated. The key to carrying out such a systematic analysis of immune system function is the development of simple, rapid assays that can be applied to a number of individual organisms from a significant number of potentially mutant lines. A number of assays have been developed by testing different aspects of the immune response, including the induction of diptericin.

For the mutant samples (in which diptericin was fused to the coding region of β-galactosidase), third instar larvae were injected with a bacterial solution of early log-phase *E. coli* using a microinjection needle and syringe (Figure 5). These larvae were allowed to recover for three hours prior to being dissected. The larvae were dissected to expose the fat body, fixed in 1.5%

glutaraldehyde in phosphate buffered saline (PBS), washed in PBS, and then infiltrated with a staining solution (10mM NaPO<sub>4</sub> buffer, 150mM NaCl, 1mM MgCl<sub>2</sub>, 3.1mM K<sub>4</sub>[FeII(CN)<sub>6</sub>], 3.1mM K<sub>3</sub>[FeIII(CN)<sub>6</sub>], and 0.3% Triton X-100; every 1 mL of solution used was mixed with 25 μL of x-gal solution). The larvae were then placed in a 37°C incubator.

## Immunohistochemistry Assay

As previously noted, upon bacterial infection, the Rel family proteins Dif and Dorsal translocate from the cytoplasm of larval and adult fat bodies to the nucleus, where they activate a series of antimicrobial peptides. A simple assay was developed in which Dif and Dorsal translocation could easily be seen under the microscope.

Third-instar larvae were injected with a culture of *E. coli* bacteria. Approximately 30–60 minutes after infection, the larvae were dissected to expose the fat body, and then fixed in 4 percent paraformaldehyde in PBS for 15 minutes. They

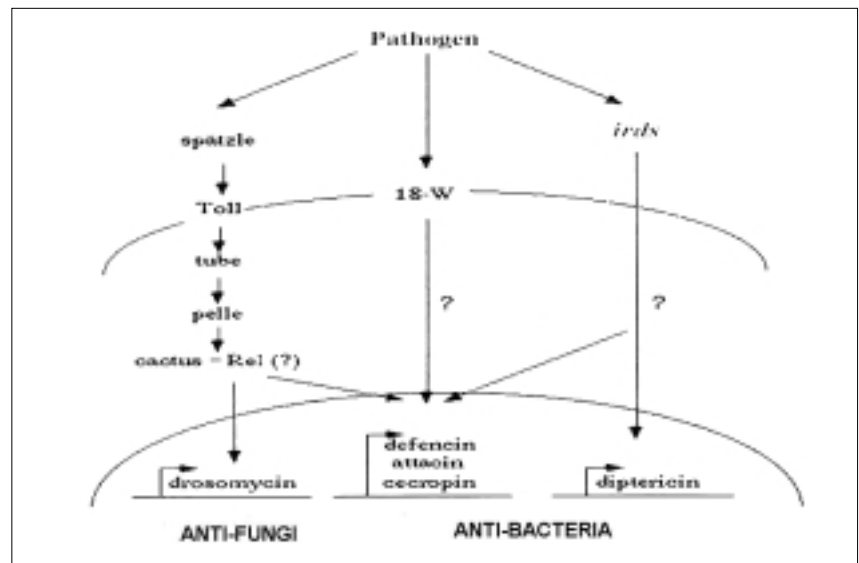


Figure 3

were then washed in PBS and infiltrated with PBTA (1% Triton X-100 and 1% BSA--Bovine Serum Albumin) in PBS for 30 minutes. The samples were later incubated with a 1:500 dilution of a rabbit anti-Dif- antibody and a 1:10 dilution of a mouse monoclonal anti-Dorsal-antibody in PBTA for one hour at room temperature (or overnight at 4°C). The samples were washed in PBTB (0.1% Triton X-100 and 0.1% BSA in PBS) and then incubated in PBTB with fluorescein isothiocyanate (FITC) goat-anti-rabbit-IgC serum and rhodamine-conjugated goat anti-mouse-IgG serum for one hour. The tissues were subsequently washed once again in PBTB and the fat bodies were dissected out completely and mounted in 90 percent glycerol in PBS for

microscopy.

## Mutant Crosses

In order to isolate any mutation, it is necessary to perform a number of crosses to map a mutant gene to a particular region of the chromosome. Once a mutant gene is isolated, it can then be cloned so that its function can be elucidated. One mutant gene of particular interest that has been chosen to be studied in depth is F34 (ird 20). As shown in Mapping Scheme 1, ird 20 virgin females were crossed to rucuca males containing a number of recessive mutations (used as markers) on their third chromosome. The resulting progeny were allowed to develop, and adult virgin females of that generation were then crossed to ruprica males containing another set of recessive mutations. (The only difference between the ruprica lines and rucuca lines is a Pr marker, which presents itself as a dominant prickly phenotype.) The progeny that resulted from this cross were then crossed to ird 20 mutants over a third chromosome balancer, TM6B

(which carries a dominant larval marker, Tubby. The resulting offspring containing the Tubby marker were tested for dipteracin activity and for nuclear translocation of Dif and Dorsal, using the aforementioned assays. Continued crossing and mapping schemes led to isolation of the ird 20 mutant to a particular region on the chromosome. A series of deficiency crossings (shown in Mapping Scheme 2) further elucidate the mutant's position. Deficiency mapping requires the use of deletions, or breaks in the chromosome, rather than recessive mutations as used in the recombination crosses.

## Control

In each assay performed in this research, the control used was the wild-type response to bacterial infection. In the dipteracin-lacZ assay, approximately three hours after infection, wild-type larvae carrying the reporter gene stained blue, indicating high levels of  $\beta$ -galactosidase activity in their fat bodies. Mutants were thus identified by a lack of staining. The immunohistochemical staining of wild-type larvae reveals nuclear translocation of both Dif and Dorsal approximately 2.5 hours after bacterial infection. Therefore, mutant lines were identified by

absence of nuclear translocation or by the presence of nuclear translocation before and after infection. Further analysis, such as Northern blots and Reverse Transcription-Polymerase Chain Reactions (RT-PCR), revealed that in wild-type flies, dipteracin is induced upon bacterial infection, while it is clearly absent in mutant samples.

## Methods of Gathering Data

The key to carrying out such a systematic analysis of immune system function is the development of simple, rapid assays that can be applied to a number of individual organisms from a significant number of potentially mutant lines. The dipteracin-lacZ assay requires that larvae be injected with an E.coli solution. Approximately 30 minutes after injection, they are dissected and stained, as described earlier. Thus, larvae are observed 2.5 hours after infection to check for staining. At this time, in wild-type larvae, the normal immune response has

been activated. The fat bodies were checked for staining, blue staining being a sign of a functioning immune system and no staining signifying the presence of a mutant gene.

The immunohistochemical staining of larvae involves observation of the nuclear translocation of the Dif and Dorsal transcription factors under

the microscope. Just as with the lacZ assay, larvae were injected, dissected, and stained. Once again, larvae were observed approximately 3 hours after bacterial infection. Fat body cells were carefully observed under the fluorescent microscope, and nuclear translocation was identified by the presence of localized fluorescent dots throughout the fat body cells (these dots, of course, being the nuclei). The mutant crosses involved a tremendous amount of patience and observation.

Approximately 2 weeks after a cross is made, the new generation will appear. Thus, in working with *Drosophila*, there is a time schedule to be followed. It was necessary to observe the flies at least once a day, noting the presence of any abnormal phenotypes and collecting virgins. Crosses made were placed in an 18° C incubator. Third-instar larvae were then used for the aforementioned assays.

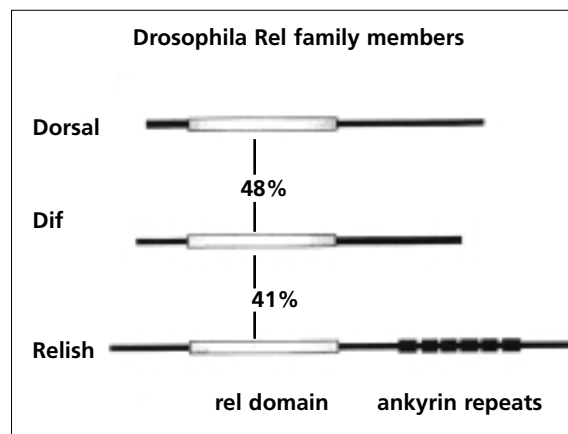


Figure 4

## Recommended Readings

1. Fearon, D.T. Seeking wisdom in innate immunity. *Nature*. July 24, 1997; 262: 323-325.
10. Nappi, A.J. The role of melanization in the immune reaction of larvae of *Drosophila* algonquin against *Pseudeucoila bochei*. *Parasitology*. 1973; 66: 23-32.

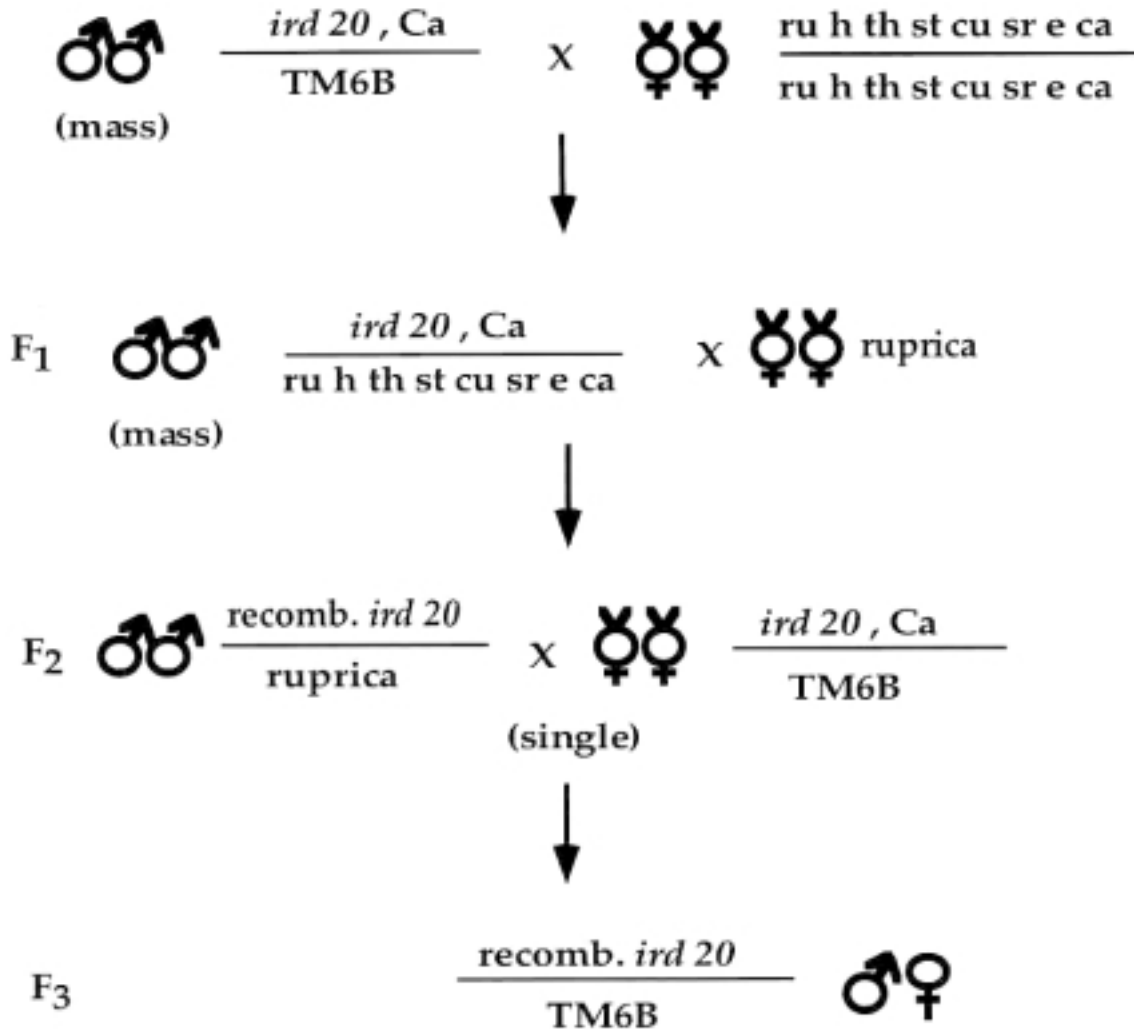
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**RESULTS:**

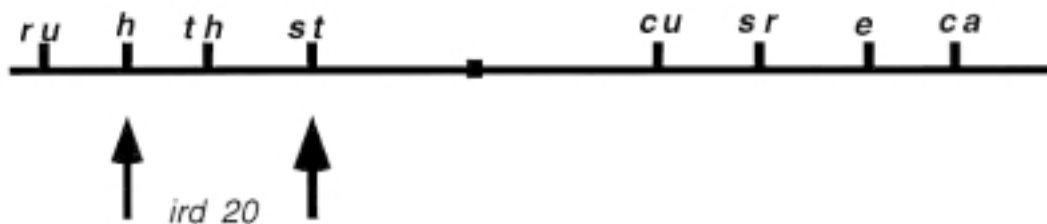
**Mapping Results For *Ird20***

*I. Recombination Mapping Scheme*



Screen third instar larvae for dipterucin expression

CHROMOSOME 3:



## Discussion

Using the dipterin-lacZ assay, mutant lines can be identified by a lack of staining. Approximately 3 hours after infection, wild-type larvae carrying the reporter gene showed high levels of  $\beta$ -galactosidase activity, while mutant larvae showed none. Wild-type larvae stain blue, positive for  $\beta$ -galactosidase activity in the fat body. This is indicative of a normal immune response. The ird 20 line failed to stain at all, and, thus, was a candidate that lacked the components necessary for this aspect of the immune response. (The position of the mutant gene can be determined using the phenotypes that were expressed in the adults of that generation.)

Among the 57 ird mutants or more than 40 ird genes (several lines fall into the same complementation groups), some are chosen to be studied in depth. These are then divided into two classes according to their Dif nuclear translocation in the fat body upon bacterial infection. The mutants that block induced nuclear localization are designated Class I mutants, while in Class II mutants, Dif is constitutively present in fat body nuclei before and after infection.

Conclusive data has been collected for one of these mutant genes, ird 20. (Mapping is under way for other ird mutant lines.) Ird 20 has been mapped on the third chromosome, between the 66D15 and 73A3-A4 regions Mapping Scheme I. (Using the genetic map method, geneticists separate the four chromosomes of *Drosophila* into 100 units, the third chromosome being of greater importance due to the multiple markers that it contains.) Ird 20 was mapped between the markers h and st on the left arm of the third chromosome. St (scarlet) expresses an eye phenotype, in which the eyes are colored scarlet, and h (hairy) expresses an abnormal bristle phenotype. ird 20 itself has no significant abnormalities expressed in its phenotype. After conducting the immunohistochemistry assay, it was found that for ird 20, Dif is constitutively nuclear before and after infection. (Wild-type larvae show translocation only after infection; ird 20 does not follow this pattern and is therefore considered a mutant.) Thus, ird 20 is one of the Class II mutants.

A series of deficiency crosses (using deletions on the chromosome rather than recessive mutations) was performed (refer back to Mapping Scheme II), and preliminary results indicate that the ird 20 mutant gene is located very close to the h marker. Further deficiency mapping and cloning of this gene will serve to elucidate its function; deficiency mapping is currently under way.

Thus, by manipulating DNA sequences in the third chromosome of the *Drosophila* genus and assessing the absence of larval immune response,

it is possible to find the essential components involved in the *Drosophila* immune system upon bacterial infection. Isolating ird 20 is just one of the steps involved in elucidating the pathways that are involved in *Drosophila* immune response. This study has isolated ird 20 to the left arm of the third chromosome, between the 66D15 and 73A3-A4 regions. Immuno-histochemical staining of ird 20 has shown that it is a Class II mutant, lacking dipterin induction upon infection. Only after cloning this gene and analyzing its molecular gene products will it be possible to elucidate its function in inhibiting immune response.

## Future Research

The definitive analysis of how these genes affect the immune response will come from the molecular analysis of their gene products. Particularly for genes that are required for dipterin induction but do not appear to affect the function of the Rel proteins, additional molecular tools are necessary to elucidate their function. Thus, the cloning of one or more of these genes will be of high priority.

## Applications

The proposed studies on the *Drosophila* immune response will provide new perspectives on the conserved innate immune response. Results should have important implications for both the mechanisms of innate immunity and for the control of insect-borne diseases. Although many aspects of the mammalian immune response have been well studied, the innate immune response has been, by comparison, almost overlooked. The specific *in vivo* roles of particular signaling pathways and transcription factors in the mammalian innate immune response are far from clear. Given the extraordinary successes in applying the results of systematic genetic and molecular analysis from *Drosophila* to the understanding of vertebrate development, it seems inevitable that this study will provide important new perspectives on the mammalian innate immune response. It is quite likely that this research will also identify new genes and regulatory networks that function in mammalian immune response.

## Acknowledgements

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