# Identification of Immune System and Response Genes, and Novel Mutations Causing Melanotic Tumor Formation in *Drosophila melanogaster*

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ABSTRACT

We are using Drosophila as a model system for analysis of immunity and tumor formation and have conducted two types of screens using enhancer detector strains to find genes related to these processes: genes expressed in the immune system (type A; hemocytes, lymph glands and fat body) and genes increased in expression by bacterial infection (type B). For type A, tissue-specific reporter gene activity was determined. For type B, a variation of enhancer detection was devised in which β-galactosidase is assayed spectrophotometrically with and without bacterial infection. Because of immune system involvement in melanotic tumor formation, a third type was hypothesized to be found among types A and B: genes that, when mutated, have a melanotic tumor phenotype. Enhancer detector strains (2800) were screened for type A, 900 for B, and 11 retained for further analysis. Complementation tests, cytological mapping, *P*-element mobilization, and determination of lethal phase and mutant phenotype have identified six novel genes, *Dorothy, wizard, toto, viking, Thor* and *dappled*, and one previously identified gene, *Collagen IV*. All are associated with reporter gene expression in at least one immune system tissue. *Thor* has increased expression upon infection. Mutations of *wizard* and *dappled* have a melanotic tumor phenotype.

BALANCING the need to identify and destroy aberrant cells and infectious organisms with the requirement to change cellular identity during normal growth and differentiation is complex and delicate, but imperative for survival. If balance is not maintained, a variety of complications can arise. When normal cells are mistaken as non-self, autoimmune disorders may develop. When cells become aberrant, tumors may form and cancer ensue. When foreign organisms overgrow, life threatening infections may occur. Central to maintaining balance is the immune system, which responds immediately and generally by the component of innate immunity and more slowly and specifically by antibody-based immunity. In recent years, the mechanisms by which the immune systems of diverse organisms accomplish these goals have been revealed to have striking similarities, particularly at the level of innate immune defense (for reviews see HOFFMANN *et al.* 1994; COLTON and RAVETCH 1995). We are using Drosophila as a model system for genetic dissection of the processes that accomplish these goals and particularly focus on the relationship between the immune system and tumor formation.

Analysis of immunity in Drosophila and other insects increasingly shows similarities with innate immunity in mammals (reviewed in KIMBRELL 1991; FAYE and HULTMARK 1993; HOFFMANN 1995). The insect immune response is both humoral, the induced production of proteins, many of which are antibacterial and abundant in the hemolymph, and cellular, the mobilization of hemocytes for phagocytosis. Hemocytes also form a melanin-containing multilayered complex that encapsulates invading organisms. The antibacterial proteins are mainly synthesized in the fat body and to a lesser extent in a subset of the hemocytes (*e.g.*, cecropins; SAMAKOVLIS *et al.* 1990). The fat body is analogous to the mammalian liver, which produces acute phase response proteins (reviewed in BAUMANN and GAULDIE 1994; STEEL and WHITEHEAD 1994). The insect antibacterial proteins can be categorized as belonging to one of several families: cecropins, attacins, lysozymes, defensins and proline rich peptides (reviewed in HULTMARK 1993; MARSH and GOODE 1994). Members of these families also share homologies with mammalian antibacterial proteins (reviewed in HETRU *et al.* 1994). Many insect antibacterial protein genes have been molecularly cloned and characterized (reviewed in HULTMARK 1993), but no mutations of these genes have been found.

Regulation of the humoral immune response in Drosophila also has homologies with the acute phase response in mammals, with both involving Rel/NF-κB family members and κB *cis*-regulatory sequences in signaling (reviewed in IP and LEVINE 1994). The Drosophila gene *dorsal-related immunity factor* (*Dif*) encodes a NF-

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κB homologue that participates in the immune response by translocating from the cytoplasm to the nucleus and binding κB motifs of antibacterial protein genes (ENGSTRÖM *et al.* 1993; IP *et al.* 1993; PETERSEN *et al.* 1995). Molecular signaling in the cellular response in Drosophila is currently more problematic than the humoral response. The phenoloxidase system is involved in the synthesis of melanin and is a candidate in the regulation of the cellular response, but the mechanism by which the cascade is initiated is unknown (reviewed in WRIGHT 1987; RIZKI and RIZKI 1990; FUJIMOTO *et al.* 1995; other insects, ASHIDA and BREY 1995; HALL *et al.* 1995). However, a novel candidate effector gene of the cellular response has recently been identified, *malvolio*, which is expressed in hemocytes and is homologous to mammalian natural resistance-associated macrophage proteins (RODRIGUES *et al.* 1995).

The development and functions of the tissues involved in the immune response of Drosophila are also not fully understood. The humoral immune system, the fat body, has been well studied in a variety of other contexts (*e.g.*, similar regulation to human liver, ABEL *et al.* 1992; yolk protein genes, ABRAHAMSEN *et al.* 1992; development, HOSHIZAKI *et al.* 1994). With respect to antibacterial protein genes, transcription is known to be induced in both larval and adult fat body cells, including the larval cells that are present transiently in the adult (SAMAKOVLIS *et al.* 1990). The cellular immune system, hemocytes and lymph glands, are less characterized. In embryos, hemocytes derive from the head mesoderm and become phagocytic, engulfing cells that have undergone apoptotic cell death (TEPASS *et al.* 1994). In larvae, hemocytes originate from the hematopoietic organ, the lymph glands (SHRESTHA and GATEFF 1982). Lymph glands derive from the lateral mesoderm (RUGENDORFF *et al.* 1994) and when fully developed, appear as five to seven paired lobes along the dorsal vessel next to the brain and ventral ganglion with pericardial cells positioned between each lobe and continuing along the length of the dorsal vessel (reviewed in RIZKI 1978). Development of the dorsal vessel and associated cells has been shown to involve the homeobox gene *tinman* (AZPIAZU and FRASCH 1993) and the pair rule gene *odd Oz* (LEVINE *et al.* 1994). Larval hemocytes can initially be classified as crystal cells or plasmatocytes, with the latter giving rise to two additional cell types, podocytes and lamellocytes (SHRESTHA and GATEFF 1982). The function of crystal cells appears to involve release of the crystalline contents to provide substrates for melanin, blood clotting or tanning of the puparium (RIZKI 1957a; RIZKI 1978). In contrast, the plasmatocyte lineage clearly functions as phagocytic cells that can be compared with vertebrate macrophages and natural killer cells (ABRAMS *et al.* 1992; PEARSON *et al.* 1995). The role of the hemocytes in tissue remodeling and the interrelationship with programmed cell death during metamorphosis is yet to be fully characterized (RIZKI 1957b). At the end of metamorphosis, the lymph glands are no longer present, and the hemocytes found in adults are generally thought to be carried over from the larval stage (see GATEFF 1978; RIZKI 1978).

The cellular immune system thus does double duty in terms of recognition and response to self *vs.* nonself in that during normal development certain self cells are phagocytosed and during infections foreign invaders are phagocytosed and encapsulated. In many mutations in Drosophila, cellular immune system function with regard to self appears to be affected (reviewed in GATEFF 1978, 1994; SPARROW 1978; WATSON *et al.* 1994). The defining characteristic of these mutations is melanotic tumors, black masses of tissue that are free floating in the hemocoel or attached to various organs. These masses are aggregates of hemocytes or hemocytes surrounding cells from other tissues. The hemocytes thus appear to form capsules and deposit melanin as a normal strain would during an immune response, but in this case the encapsulation is directed against the organism itself. These mutations can be viable or lethal and may or may not show an overgrowth phenotype as well. Mutations that do not show overgrowth are designated as melanotic tumor mutations or melanotic pseudotumor mutations (reviewed in SPARROW 1978). Mutations that also show overgrowth are oncogenic or tumor suppressor mutations, and the genes that these mutations identify are part of the growing list that has established Drosophila as a model system for the study of cancer (reviewed in GATEFF 1994; WATSON *et al.* 1994; HARRISON *et al.* 1995; LUO *et al.* 1995).

A genetic dissection of the processes involved in immunity and tumor formation clearly requires the identification of many more genes. At this time, however, genetic groundwork still needs to be done as many fundamental questions are unanswered and more tools for analysis of the processes are required. As a step to address this need, we have used *P*-element enhancer detector strains to conduct two types of screens. In the first, enhancer detector strains were screened for tissue specifc expression of *lacZ* in the immune system. In the second, a new type of screen using enhancer detector strains was designed to detect increased expression of *lacZ* in response to bacterial infection. The first screen selects for genes expressed in the hemocytes, lymph glands and fat body. The second screen selects for genes increased in response to bacterial infection, regardless of tissue specificity. Inclusive in this experimental plan is that genes from both screens will overlap with a third category, genes that lead to tumor formation when mutated. We present these screens and the identification of six previously undescribed genes that include representatives of all three categories targeted.

## MATERIALS AND METHODS

**Stocks:** Enhancer detector stocks were from the collections of TÖRÖK *et al.* (1993), the Indiana Drosophila Stock Center and MICHAEL STERN (Rice University). The deficiency stocks

**TABLE 1**

**Deficiencies used in this study**

| Deficiency | Cytological breakpoints |
|---|---|
| *Df(2L)fn27* | 35B1-35D1-2 |
| *Df(2L)r10* | 35E1-2; 36A6-7 |
| *Df(2L)osp29* | 35B1-3; 35E6 |
| *Df(2L)A48* | 35B2-3; 35D5-7 |
| *Df(2R)X1* | 46C; 47A1 |
| *Df(2R)X3* | 46C; 46E1-2 |
| *Df(2L)dp-h25* | 24E2-4; 25B2-5 |
| *Df(2L)sc19-6* | 24F1-2; 25C3-5 |
| *Df(2L)sc19-1* | 24D4-5; 25C8-9 |
| *Df(2R)pk78k* | 42E3; 43C3 |
| *Df(2R)ST1* | 43B3-4; 43E18 |
| *Df(2R)cn83c* | 43C5-D1; 44B6-C1 |

Deficiency stocks can be obtained from the Indiana Drosophila Stock Center and references from LINDSLEY and ZIMM (1992).

used in this study are shown in Table 1. The Dipt2.2-*lacZ* transgenic strain was provided by JEAN-MARC REICHHART and JULES HOFFMANN (CNRS Strasbourg; REICHHART *et al.* 1992). The *CyO, P{Antp-lacZ}* strain was provided by YLVA ENGSTRÖM (University of Stockholm). The *CyO, A109.1F2* strain was received from WALTER GEHRING's laboratory (Biozentrum, Basel; WILSON *et al.* 1989). The *tu bw* and *tu g* stocks were from the Bowling Green Stock Center. Other stocks used in this study were from the Indiana Drosophila Stock Center. Mutations are described in LINDSLEY and ZIMM (1992).

**β-galactosidase staining of embryos, larvae and adults:** The procedures were essentially those of protocols 75 and 77 of ASHBURNER (1989). Staining was done for long periods, overnight, to detect all possible tissues with *lacZ* expression.

**Assay for changes in level of β-galactosidase activity:** A spectrophotometric assay of level of β-galactosidase activity from siblings with and without an infection was a modification of protocol 133 of ASHBURNER (1989). Bacteria for injection (*Enterobacter cloacae* B12; FLYG *et al.* 1987) was grown to log phase in LB medium, concentrated by centrifugation, and injected into third instar larvae or adults by the tip of a fine needle dipped into the bacterial solution. Injected larvae were stored at 25° for 5–6 hr, and adults for 6 hr at 25° or overnight at 18° before homogenization and spectrophotometric assay. Each sample had two larvae or adults, and the adult sample contained one male and one female. Each sample was divided in half, *i.e.*, duplicate readings, and represent 80% of one fly (or larva). Samples were read and processed by a Thermomax microtiter plate reader and the Softmax computer program. The OD $574_{nm}$ range is from 0 to a maximum of 4, and at 37° β-galactosidase activity is a linear progression over several hours. Control flies for background activity and homogenate standardization were Canton S. Control flies for maximum inducibility were Dipt2.2-*lacZ* flies, which have 2.2 kb of 5′ flanking sequences of the *Diptericin* gene fused with the coding region of *lacZ* (REICHHART *et al.* 1992). See Figure 6 of RESULTS for graph of control data on adults.

***In situ* hybridization to polytene chromosomes:** Flies heterozygous for the enhancer detector insertion were crossed to *y w; CyO/S sp Bl* and progeny *y w; P{lacZ,w+}/S sp Bl* crossed together. Salivary glands of their progeny were dissected in Ringer's saline and fixed with acetate. Pretreatment, hybridization and detection were essentially as in KANIA *et al.* (1995).

**Isolation of revertants:** The isolation of revertant chromosomes was by mobilization of the *P* element with Δ2-3 transposase as described in TÖRÖK *et al.* (1993).

**Lethal phase and phenotype:** *P*-element containing chromosomes were balanced with *CyO, P{Antp-lacZ}* to allow for identification of homozygous mutant embryos. *P*-element containing chromosomes were also balanced with *y w; CyO,y+* to allow for identification of homozygous mutant larvae.

## RESULTS

Two types of screens were conducted using enhancer trap strains. One screen selects for possible immune system genes by assaying for tissue specific expression of *lacZ* in the immune system, an enhancer detector screen as in BELLEN *et al.* (1989) and BIER *et al.* (1989). The second screen selects for infection response genes by assaying for a change in *lacZ* expression after bacterial infection. These results are presented under the headings of immune system enhancer detector screen and infection inducible enhancer detector screen.

**Immune system enhancer detector screen:** To identify enhancer detectors with immune system reporter gene expression, 2800 enhancer detector strains were screened for tissue specific β-galactosidase activity in hemocytes, lymph glands and/or the fat body. Embryos of 2000 strains from the collection of TÖRÖK *et al.* (1993) and third instar larvae of 800 strains from the collections of the Indiana Drosophila Stock Center and MICHAEL STERN were screened. Eighteen strains were selected and scored further for tissue specific reporter gene activity throughout development (Table 2; DEMEREC 1950; CAMPOS-ORTEGA and HARTENSTEIN 1985; BATE and MARTINEZ-ARIAS 1993).

Strains with many sites of expression in addition to the immune system and strains with *P*-element insertions that were not stable or readily separable from a second insertion were discontinued. The remaining strains were tested further, beginning with complementation of their mutations, and cytological mapping of the *P*-element inserts. To test that the lethal mutations were caused by the insertions, the *P* elements were mobilized and the resultant strains tested for reversion to viability. The time of lethality and phenotype were determined, and a test for response to infection was conducted. The results of these enhancer detector strain analyses are presented in the categories of lymph gland, hemocyte and fat body, and fat body enhancer detector strains.

*Lymph gland enhancer detector strains:* Three insertions have identified three new genes that, following the Wizard of Oz theme of *tinman* (AZPIAZU and FRASCH 1993) and *odd Oz* (LEVINE *et al.* 1994), we have named *Dorothy* (*Dot*), *wizard* (*wiz*) and *toto* (*toto*).

The 80/12 strain shows a β-galactosidase pattern in embryos restricted to the lymph glands and pericardial cells and identifies the *Dorothy* gene (Figure 1A and Table 3). In larvae β-galactosidase expression is in the lymph glands and pericardial cells, and a few cells in the proventriculus (Figure 2A and Table 3). The *P*-element insertion maps to 24A. To determine whether the *P* element causes lethality, we mobilized it with Δ2-3 transposase,

**TABLE 2**

**Tissue specific staining of β-galactosidase**

| Stock[a] | Embryonic | Larval[b] | Adult[c] |
|---|---|---|---|
| 80/12 (*Dorothy*) | lg, pc | lg, pc, p[d] | bg[e] |
| 108/11 (*wizard*) | lg, o, ps, hd | lg, o, m, ma | o, m[e] |
| 3-50 (*toto*) | mg, ps | lg, sg, m | bg[e] |
| 138/1 | lg, fb, ps | lg, fb, m, cns, mt[e] | fb, b, m |
| 115/33 | fb, mg, pd | lg, fb, cns, mt, tt, m | fb, mt |
| 90/5 | lg, mg[g], hg | lg, fb, sg, cns, p, m, ma, h | c, es, m? |
| 134/20 ($Cg25C^{GDB}$) | h, fb | fb, cns | fb |
| 71/38 ($vkg^{BLK}$) | h, fb | fb | fb, o? |
| 95/11 ($vkg^{ICO}$) | h, fb | fb, cns | fb |
| 138/2 ($vkg^{RML}$) | h, fb | fb, cns | fb |
| 167/21 ($vkg^{SAK}$) | h, fb | fb | fb |
| 135/17 (*Thor*) | cns[f] | fb, rg, sg, es, p[d], g, m, mt, ma | fb, es, mt, e, m?, sg? |
| 142/2 ($dpld^{EJL}$) | rg | lg, rg, fb, o, mt, m | fb, o, mt, m, ca, cc? |
| 97/16 ($dpld^{MLB}$) | rg, mg, pd | fb, rg, o, m, mt | fb, o, mt, m, ca, cc? |
| 72/39 | fb, m, cns? | fb, cns, m, mt | fb, b, mt, e, m |
| 2130 | complex[h] | fb, b, m | ND |
| 136/39 | complex[h] | fb, cns, d, p, m, mt | ND |
| P160 | ND | fb, b, m, g | fb, b, mt, m? |
| P2235 | ND | fb[e] | fb |

b, brain; bg, background; c, cardia; ca, corpus allatum; cc, corpus cardiacum; cns, central nervous system; d, imaginal discs; e, eye; es, esophagus; fb, fat body; g, gastric caecae; h, hemocytes; hd, head; hg, hindgut; hi, hindintestine; lg, lymph glands; m, midintestine; ma, mouth armature; mg, midgut; mt, Malpighian tubules; o, oenocytes; p, proventriculus; pc, pericardial cells; pd, anal pads; ps, posterior spiracles; rg, ring glands; sg, salivary glands.

[a] Data in parentheses are names.

[b] Background staining in wild-type Canton S and strains screened is garland cells, anterior spiracles, a section of the intestine, and variable staining at the cuticle, part of the first pair of lymph glands and the cytoplasm of hemocytes. Only strains with strong nuclear staining of the hemocytes were scored as positive. Scoring of the gonads is not included.

[c] Background staining in wild-type Canton S and strains screened is pericardial cells, nephrocytes, a section of the intestine, and variable staining of the antennae and legs. Scoring of the reproductive system is not included.

[d] A subset of cells in the proventriculus.

[e] Staining partially characterized.

[f] A few cells also stain at the anterior tip and in unidentified cells in region of midgut.

[g] Several additional unidentified cells scattered in region of amnioserosa and midgut.

[h] A variety of staining cells that includes the fat body.

but excision did not revert the lethality (see MATERIALS AND METHODS). Removal of lethals by recombination showed that the *P*-element insertion at 24A does not affect viability. Genomic DNA flanking the insertion was isolated and hybridized to whole mount embryos, and this showed an RNA pattern identical to the β-galactosidase pattern in Figure 1A (Z. ZHOU and D. KIMBRELL, unpublished results). Hence, 80/12 identifies a lymph gland and pericardial cell specific gene. In homozygous 80/12 adult flies infected with bacteria, the level of β-galactosidase does not change and the viability of infected flies is not affected (data not shown). We are also using this strain to investigate further the development of lymph glands, which, as shown in Figure 1A, start as one pair in embryos. The genes of the *Bithorax Complex* (*BXC*) regulate segmental identity in the abdomen and posterior thoracic segments (LINDSLEY and ZIMM 1992) and thus were chosen as candidates to test for regulation of lymph gland development. We have found that the *BXC* does regulate lymph gland identity, as more extreme mutation of *BXC* causes development of more lymph glands and fewer pericardial cells. An example is shown in Figure 3C, for 80/12; *bithorax Ultrabithorax* (*bx Ubx*) embryos. The *BXC* domain that includes *bx Ubx* regulates identity in parasegments 5 and 6. Thus in the 80/12; *bx Ubx* embryos, transformation of a more posterior identity, pericardial cells, to a more anterior identity, lymph glands, has occurred, resulting in the formation of two pairs of lymph glands instead of the normal one pair. These results with *BXC* are consistent with those reported previously by MASTICK *et al.* (1995), in which they selected for target genes regulated by *Ubx* and found that lymph gland morphology and expression of a target gene extended further along the dorsal vessel in $Ubx^{9.22}$ than in wild type.

The *wizard* gene is defined by a lethal mutation on the 108/11 insertion chromosome. β-galactosidase is expressed in the embryonic lymph glands, oenocytes and stomatogastric nervous system (Figure 1C; larvae and adults Table 2). The lethality could not be reverted

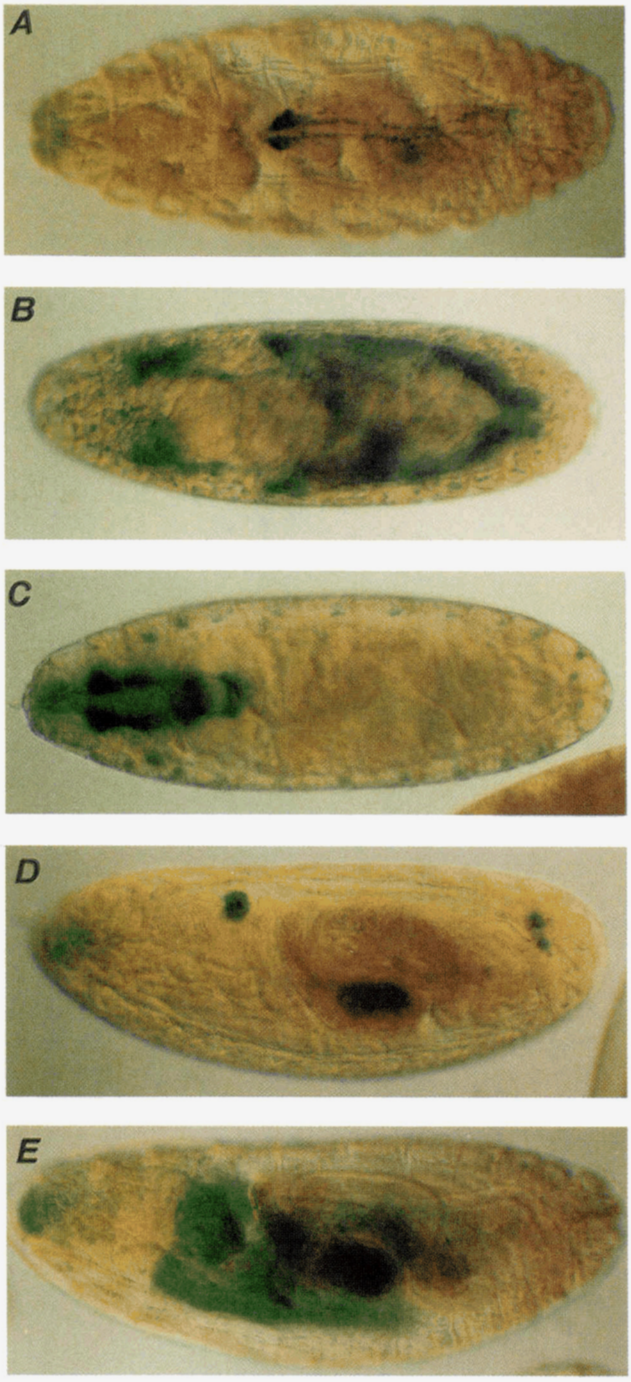


FIGURE 1. —Stage 17 embryos stained for β-galactosidase activity. Staining tissues are listed for each strain. Photos are of embryos heterozygous for the enhancer detector containing chromosome and the *CyO* balancer. (A) *Dorothy*: lymph glands and pericardial cells. (B) *viking*: fat body and hemocytes. (C) *wizard*: lymph glands, stomatogastric nervous system and oenocytes. (D) *dappled*: ring glands and anal pads. (E) *Thor*: central nervous system. Dorsal (A–C) and lateral (D and E) view. Anterior, left; posterior, right.

by *P*-element mobilization, even after recombination to remove lethals. However, the correspondence between the cytological location of the insert, 35E, and lethality mapping into overlapping deficiencies in the same region suggests that the lethality is the result of the *P*-element insertion (Table 3). The time of lethality and phenotype were thus determined for heterozygotes of 108/11 and deficiencies. Approximately 80% of both 108/11/*Df(2L)osp29* and 108/11/*Df(2L)A48* embryos die before hatching and appear normal. The remaining 108/11/*Df(2L)osp29* and 108/11/*Df(2L)A48* embryos hatch and are found to have small melanotic tumors scattered throughout the body (Figure 4A). A few of these tumors are attached to the epidermis, and the rest are free floating in the hemocoel; correspondingly, the position of these tumors is highly variable in different larvae. These tumors do not grow larger than the sizes depicted in Figure 4A, and these larvae die during the first instar.

The *toto* gene is defined by the lethal mutation of the insertion in strain 3-50. The insertion is located at 46F, included within the region defined by deficiencies *Df(2R)X1* and *Df(2R)X3*, and the lethality is revertible by excision of the *P* element (Table 3). The lethal phase is during embryogenesis, and the mutant embryos appear normal (Figure 3A). Since the β-galactosidase staining of lymph glands does not start until the larval stage and the lymph glands form normally in mutant embryos, lethality during embryogenesis probably is unrelated to the lymph glands. In larvae that are heterozygotes (*CyO/3-50* or *+/3-50*), however, there is a dominant phenotype of enlarged lymph glands (Figure 2B). Counting of circulating hemocytes in these larvae compared to wild-type larvae shows that this enlargement does not correlate with an increase in the number of blood cells in the hemolymph (data not shown).

*Hemocyte and fat body enhancer detector strains:* All of the five strains with β-galactosidase expression in embryonic hemocytes, 134/20, 71/38, 95/11, 138/2 and 167/21, also have expression in the embryonic fat body (Figure 1B). As larvae, all have β-galactosidase activity in the fat body (Figure 2E) and did not stain above background in the larval hemocytes (not shown). All of these strains also have insertions at cytological location 25C, and all of these insertions are associated with lethality that is revertible (Table 3). This cytological location and tissue specificity suggested that these might be insertions into the *Collagen IV* gene located at 25C (*Cg25C*) (NATZLE *et al.* 1982; LINDSLEY and ZIMM 1992). WILSON *et al.* (1989) identified an enhancer trap insertion, A109.1F2, into the promoter region of *Cg25C* in the balancer chromosome *CyO*. Complementation tests among the *CyO, A109.1F2* chromosome and the insertion bearing chromosomes of this screen identified two complementation groups. Insertion 134/20 does not complement *CyO, A109.1F2*, but does complement 71/38, 95/11, 138/2 and 167/21. Insertions 71/38, 95/11, 138/2 and 167/21 complement *CyO, A109.1F2* and do not complement each other (Table 3). To test the apparent identity of 134/20 as an insertion affecting *Cg25C*, the A109.1F2 insertion was mobilized by crossing to a source of Δ2-3 transposase and the resultant *CyO* chromosomes were tested for reversion

**TABLE 3**

**Immunity and tumor formation genes**

| Category | Name | Cytological location | Complementation[a] | Reversion[b] | Lethal phase[c] | Lethal phenotype | Infection inducible[d] |
|---|---|---|---|---|---|---|---|
| Lymph gland | *Dorothy (Dot)* | 24A | NA | No[e] | NA | NA | No |
| | *toto (toto)* | 46F | + *Df(2R)X3*<br>− *Df(2R)X1* | Yes | e | None | No |
| Tumor formation | *wizard (wiz)* | 35E | + *Df(2L)r10*<br>+ *Df(2L)fn27*<br>+ $sna^{18}$<br>− *Df(2L)osp29*<br>− *Df(2L)A48* | No | e-l | Melanotic tumors | No |
| Hemocytes and fat body | *Collagen (Cg25C)* ($Cg25C^{GDB}$) | 25C | + *Df(2L)dp-h25*<br>+ *mmy*<br>− *Df(2L)sc19-6*<br>− *Df(2L)sc19-1*<br>− *A109.1F2* | Yes | e-l | Newly hatched are rounded, slow moving, short lived | No |
| | *viking (vkg)* ($vkg^{BLK}$) | 25C | + *Df(2L)dp-h25*<br>+ *A109.1F2*<br>− *Df(2L)sc19-6*<br>− *Df(2L)sc19-1* | Yes | e-l | Same as above | No |
| | ($vkg^{ICO}$) | 25C | + *Df(2L)dp-h25*<br>+ *A109.1F2*<br>+ *mmy*<br>− *Df(2L)sc19-6*<br>− *Df(2L)sc19-1* | Yes | e-l | Same as above | No |
| | ($vkg^{RML}$) | 25C | + *Df(2L)dp-h25*<br>+ *A109.1F2*<br>− *Df(2L)sc19-6*<br>− *Df(2L)sc19-1* | Yes | e-l | Same as above | No |
| | ($vkg^{SAK}$) | 25C | + *Df(2L)dp-h25*<br>+ *A109.1F2*<br>− *Df(2L)sc19-6*<br>− *Df(2L)sc19-1* | Yes | e-l | Same as above | No |
| Fat body and ring gland | | | | | | | |
| Infection inducible | *Thor (Thor)* | 23F-24A | NA | No[e] | NA | NA | Yes |
| Tumor formation | *dappled (dpld)* ($dpld^{MLB}$) | 43C | + *Df(2R)pk78k*<br>+ *tu bw*<br>+ *tu g*<br>− *Df(2R)ST1*[f]<br>− *Df(2R)cn83c*[f] | ND | NA | NA | No |
| | ($dpld^{EJL}$) | 43C | + *Df(2R)pk78k*<br>+ *tu bw*<br>+ *tu g*<br>− *Df(2R)ST1*<br>− *Df(2R)cn83c* | Yes | l | Melanotic tumors, aberrant fat body and gut | No |

Data in parentheses are abbreviations: NA, not applicable; ND, not done.

[a] +, complements; −, fails to complement.

[b] Yes, reversion of lethality in jump start male progeny; no, failure of reversion of lethality in 2000 jump start male progeny.

[c] e, embryonic; l, larval; a, adult.

[d] Yes, β-galactosidase level increased by bacterial infection in adults; no, β-galactosidase level not changed by bacterial infection in adults.

[e] Second chromosome lethal(s) not associated with the *P*-element insertion was removed by recombination and the strain established as a homozygous viable enhancer trap insertion stock.

[f] Survival of heterozygotes with 100% melanotic tumor formation.

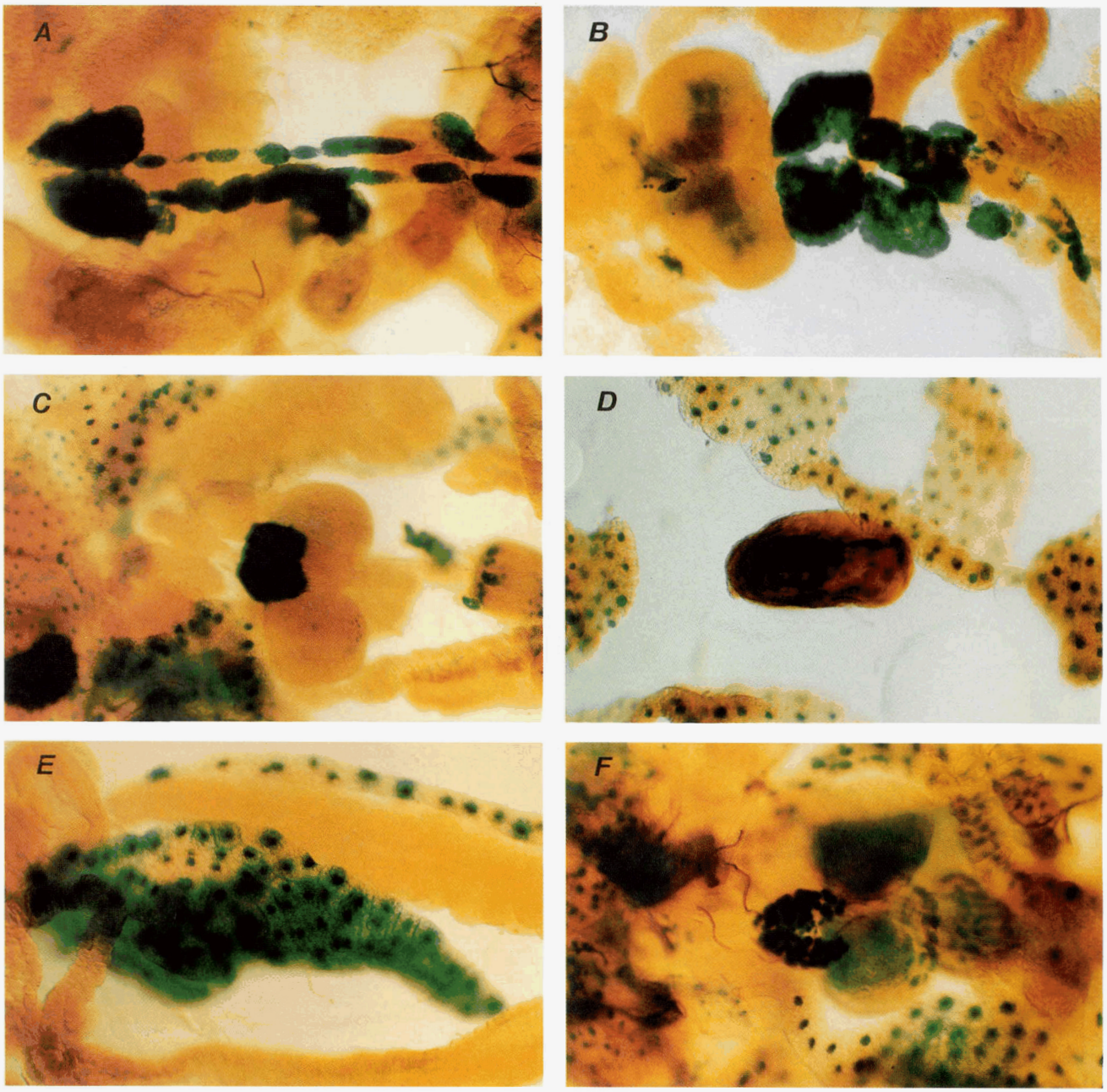


FIGURE 2.—Third instar larval tissues stained for β-galactosidase. Photos are of larvae heterozygous for the enhancer detector containing chromosome and the *CyO* balancer, except for D. (A) *Dorothy* showing staining in the lymph glands and pericardial cells. (B) *toto* with enlarged, stained lymph glands. (C) *dappled* expression in the ring glands, center of photo and over nonstaining brain lobes, and fat body. (D) Tumor from a $dappled^{MLB}$ mutant larva and staining of the fat body. (E) *viking* staining in the fat body, near the nonstaining salivary glands. (F) *Thor* staining in the ring glands, fat body, gut and part of the central nervous system. (A–F) Dorsal view. Anterior, left; posterior, right.

to viability when heterozygous with insertion 134/20. As excision of A109.1F2 produced a chromosome that complements 134/20, we conclude that 134/20 is an insertion affecting *Cg25C*. The time of lethality of 134/20 homozygotes is embryonic to first instar, and investigation of 134/20 homozygous embryos showed no dramatic defects (Figure 3B), but only ~3% survive to hatching. Those that do hatch are small, rounded, slow moving and short lived. The four remaining insertions at 25C also have the same lethal phase and phenotype as the 134/20 insertion strain. These five insertions thus define two loci at 25C: the previously described *Cg25C* and a novel locus. An alternative explanation of complex complementation for *Cg25C* is unlikely, as in subsequent experiments we isolated genomic DNA from the sites of *P*-element insertion of all of the novel locus strains that does not hybridize with *Cg25C* genomic DNA, but does hybridize with RNA in hemocytes and fat body of whole mount embryos (J. CHEN and D. KIMBRELL, unpublished results). Hence, there are two different transcripts encoded at 25C, both of which are expressed in the fat body and hemocytes; both genes

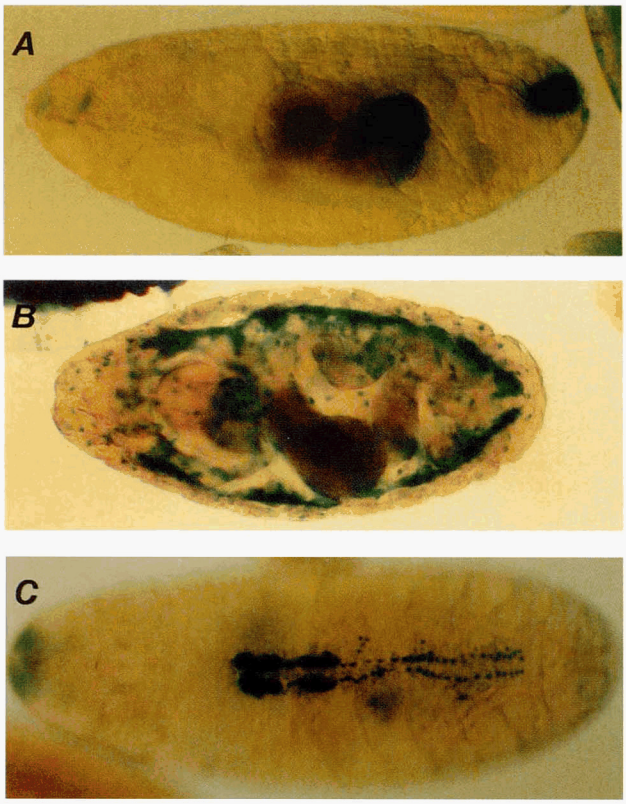


FIGURE 3.—$\beta$-galactosidase staining of mutant embryos. (A) Staining of *toto* mutant embryos does not show abnormalities. (B) Staining of $Cg25C^{GDB}$ shows that the hemocytes and fat body have formed, and the embryo is small and rounded; 3% of these embryos hatch. (C) Staining of *Dot; bx Ubx* embryos shows an increase in the number of lymph glands. Compare with Figure 1A. (A) Lateral and (B and C) dorsal view. Anterior, left; posterior, right.

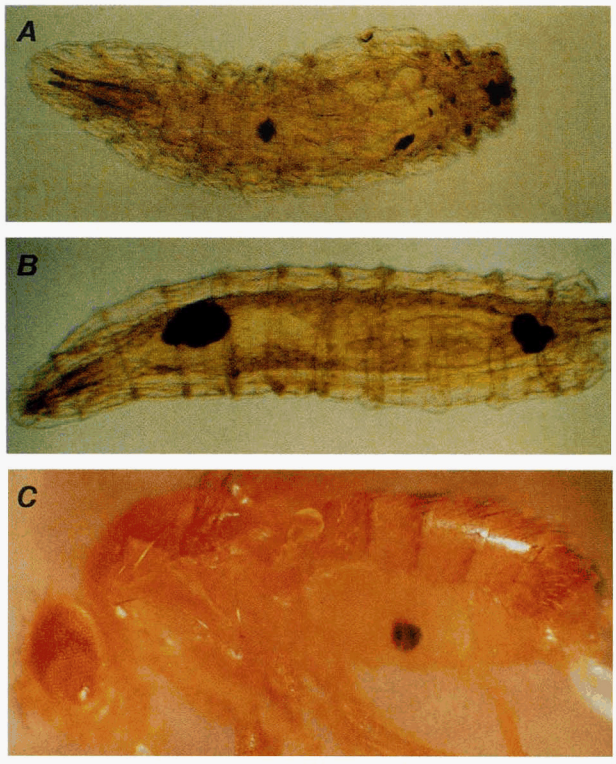


FIGURE 4.—Mutant phenotypes of larvae and adults. (A) Phenotype of multiple melanotic tumors in first instar larvae of *wiz* heterozygous with *Df(2L)osp29* or *Df(2L)A48* and $dpld^{EJL}$. Sample shown is $dpld^{EJL}$. (B) Phenotype of $dpld^{EJL}$ homozygotes can also be fewer and larger melanotic tumors, with lethality in the second instar. (C) Melanotic tumor in the abdomen of a $dpld^{MLB}$ adult. Anterior, left; posterior, right.

are essential and identified by specific mutations. The expression in immune tissues that are involved in both attack and defense, and the persistence of the few survivors as larvae has lead us to name this new gene *viking*.

*Fat body enhancer detector strains:* Strains 97/16 and 142/2 have $\beta$-galactosidase expression that includes the fat body, but not hemocytes. Both of these strains have reporter gene expression that changes from the ring glands in embryos to the ring gland, fat body and additional tissues in larvae (Figure 1D; Table 2). The 97/16 strain is homozygous viable and has melanotic tumor formation in 100% of larvae and adults (Figures 2D and 4C). The tumors in adults are in the abdomen and occasionally in the head and thorax as well. Complementation tests identified lethal insertion 142/2 as not complementing the 97/16 insertion: heterozygotes of insertions 142/2 and 97/16 survive and have 100% tumor formation as in 97/16 homozygotes. Both the 97/16 and 142/2 insertions localize cytologically to 43C, are included in *Df(2R)ST1* and *Df(2R)cn83c*, and complement other melanotic tumor mutations mapped to this region of the second chromosome (Table 3). The 142/2 lethality reverts to viability and wild-type phenotype when the *P* element is excised. 142/2 homozygotes die as larvae with melanotic tumors (Figure 4, A and B). The melanotic tumors gradually become visible as the larvae grow after hatching; lethality is observed during the first and second instar, and none of the lethal larvae reach the wandering stage of the third instar. The tumors are free floating, as seen in Figure 2D, and the size of the tumors at the time of lethality is not larger than the example shown in Figure 4B. The cells of melanized tumors are not accessible to staining for $\beta$-galactosidase activity or visual identification; however, since the lymph glands and hemocytes appear normal, the cells in the interior are unlikely to be hemocytes or lymph gland cells. Probably normal hemocytes are responding to other cells that are aberrant, and the most likely candidates are gut and fat body cells, as these often have abnormal morphology (Figure 5). Because of the melanotic tumor phenotype, *dappled* is the name we have given the gene identified by these strains.

**Infection inducible enhancer detector screen:** To identify immune response genes, a new type of screen using enhancer detectors was designed. Levels of $\beta$-galactosidase can be determined spectrophotometrically, and this has been used to describe the induction of heat shock promoter-*lacZ* fusions (SIMON and LIS 1987; ASHBURNER 1989). Varying this method, we as-

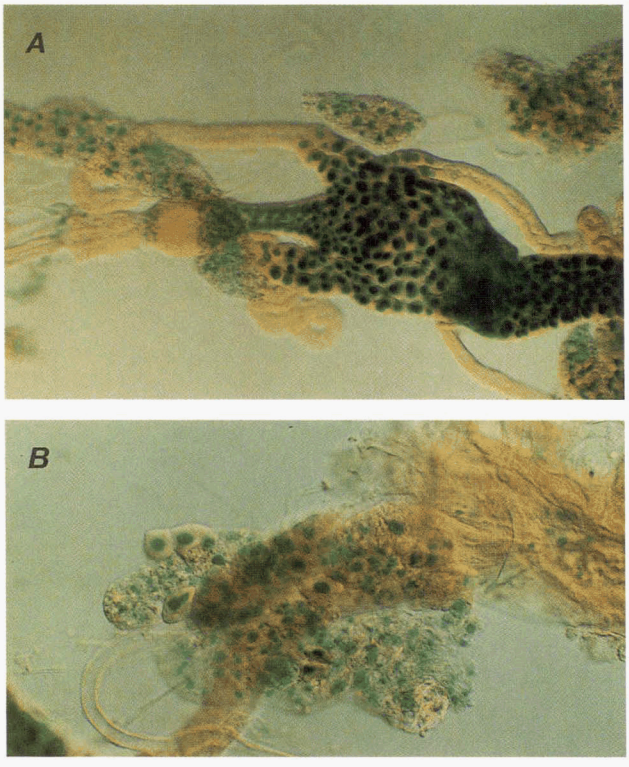


FIGURE 5.—Mutant phenotype of $dpld^{EJL}$ tissues. (A) β-galactosidase staining of $dpld^{EJL}$ shows a distended and misshapen midintestine. This region is approximately twice the normal size. The gastric caecae, the nonstaining tendrils extending from this part of the midintestine, are also elongated two- to threefold. (B) β-galactosidase staining of $dpld^{EJL}$ shows a cluster of aberrant fat body cells. Fat body cells in the larva shown in A are also irregular, but less so than in this example.

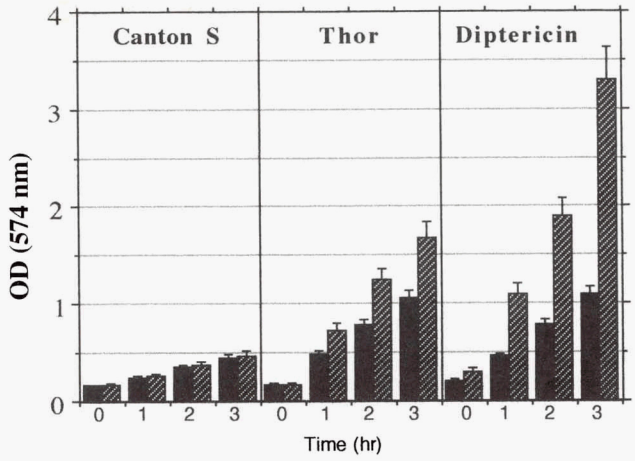


FIGURE 6.—Infection inducibility. β-galactosidase levels of homogenates of adults with (infected, ▨) and without (control, ■) bacterial infection were assayed spectrophotometrically over a 3-hr time period. Shaded columns show the mean and bars the standard deviation. Average sample size is 50. The Canton S stock provides a negative control for induction and a standard for endogenous activity. The Diptericin stock has the Dipt2.2-*lacZ* transgene and provides a positive control for inducibility. Assays for *Thor* are for heterozygotes of 135/17 with *CyO* and show approximately a twofold increase compared with approximately a threefold increase for Diptericin. See MATERIALS AND METHODS for details.

sayed for induction or suppression of enhancer detectors in response to bacterial infection. The advantage of this approach is that it does not select for a presumed phenotype associated with mutation of an infection response gene, and it is also unbiased with regard to tissue-specific expression. As described in MATERIALS AND METHODS, the assay compares the level of β-galactosidase in siblings with an infection to siblings without an infection. Strains showing an increase in β-galactosidase upon infection in initial screening were retained for further testing. Enhancer detectors indicating genes in the novel category of suppressed in response to infection were also a goal of this screen. However, since β-galactosidase is stable, a decreased response may be possible to detect only if the decrease is substantial compared with the level of β-galactosidase accumulated before infection. A total of 900 enhancer detector strains were assayed, 600 as adults and 300 as larvae and adults from the three collections also assayed for tissue specific reporter gene expression. An increased response was confirmed in one strain. No strain was found with a decreased response. Another possibility in this screen is an inducible lethal, *i.e.,* a gene that is not essential except for fighting infection. This category could be screened for among the viable enhancer detector strains tested, ~300, but scoring of relative viabilities did not indicate this type of candidate.

One known candidate for inducibility is based on experiments in the mouse that show increased transcription for xanthine dehydrogenase in response to interferon and its inducers, *e.g.,* bacterial lipopolysaccharide (TERAO *et al.* 1992). Since many of the enhancer detector strains that we assayed carry *rosy*+, the gene for xanthine dehydrogenase, our experiments incidentally included an indirect test of *rosy*. Since the reporter gene of these strains was not inducible, either xanthine dehydrogenase is not inducible in Drosophila or the construct does not have the appropriate flanking sequences for induction.

An increased level, approximately a doubling, of β-galactosidase in response to a bacterial infection was found in adults of strain 135/17 (Figure 6). A similar increase was found for larvae (data not shown). The cytological location of the insert is 23F-24A. The lethality was not revertible by *P*-element excision. Removal of lethals by recombination revealed that the insertion does not affect viability. Homozygous larvae and adults infected with bacteria also survive. The profile of β-galactosidase activity changes during development in that embryonic expression is mainly in the central nervous system (Figure 1E), and larval and adult expression is in many additional tissues, mainly the fat body, gut and ring gland (Table 2 and Figure 2F). This homozygous viable strain thus has reporter gene expression that increases upon infection and identifies the response gene we have named *Thor*, after the Nordic legendary character that was often called upon to use his hammer to protect mankind from harm.

## DISCUSSION

The novel genes *toto*, *Dorothy* and *wizard* have been identified as members in the category of hematopoietic organ reporter gene expression. *toto* is distinguished in having lymph gland staining only in the larval stage and in having enlarged lymph glands in *toto* heterozygotes, possibly indicating a gain of function mutation. The most restricted expression, lymph glands and pericardial cells, is seen in *Dorothy* embryos. Using *Dorothy*, the *Bithorax Complex* has been shown to be involved in regulating lymph gland and pericardial cell identity, and this supports the previous similar results and conclusions of MASTICK *et al.* (1995) using an *Ultrabithorax* target gene. *tinman* is known to be necessary for pericardial cell development, but not for lymph gland development (AZPIAZU and FRASCH 1993). The role of *Dorothy* in both of these structures is not yet known, and will require molecular characterization of the gene and *P*-element mobilization to produce null mutations or deletions. One of the goals of this study is to provide tools for future studies, and in this respect *Dorothy* may provide a useful promoter, to allow for production of a variety of transgenic constructs. *wizard* confirms the prediction that a subset of genes selected for immune system expression would also have a mutant phenotype of melanotic tumors and is discussed below with the gene *dappled*.

Both the novel *viking* and the previously identified *Collagen IV* were selected based on hemocyte and fat body specificity. Collagen is an interesting finding with regard to tumor formation as collagen is broken down during tumor invasion in humans, and WOODHOUSE *et al.* (1994) have determined that Drosophila is similar to humans in having increased type IV collagenase associated with invasive tumors. Our molecular characterization of *viking* has not ruled out the possibility that *viking* encodes a previously undescribed collagen or a collagen homologue (J. CHEN and D. KIMBRELL, unpublished results). If *viking* encodes another collagen IV, this would make Drosophila similar to *Caenorhabditis elegans* and humans in that both have two type IV collagen genes (*C. ELEGANS*, reviewed in KRAMER 1994; human promoter, FISCHER *et al.* 1993). A collagen homologue such as collectin is also an interesting possibility, as collectins are a collagenous C-type lectin of innate immune defense in mammals and birds (reviewed in HOLMSKOV *et al.* 1994).

The fat body, rather than the lymph glands or hemocytes, has been the common immune tissue with reporter gene expression in the strains we have identified. *Thor*, which was selected for inducibility rather than tissue specificity, also has fat body expression. *Thor* is probably not an inducible antibacterial protein gene, as according to the reporter gene it would be expressed without an infection and this is not the case for the known Drosophila inducible antibacterial protein genes. The expression of *Thor* is more analogous to the silkmoth gene encoding hemolin, the nonantibacterial immunoglobulin superfamily member, as hemolin is present without an infection and increases in response to an infection (SUN *et al.* 1990). Although the number of induced (or repressed) immune response genes is unknown, clearly a variety of additional genes remain to be identified. For example, the induced proteins visible in the gels of the early experiments by ROBERTSON and POSTLETHWAIT (1986) have only been partially correlated with known products. A more sensitive technique, PCR-based differential display, reveals more candidates for inducibility and has recently allowed identification of the antibacterial protein gene *Attacin A* (ÅSLING *et al.* 1995).

An immune response gene null mutation would be lethal if the gene were also expressed without an infection and served an essential function or if it were a critical gene in the immune response. The latter would be an inducible lethal, *i.e.*, lethality conditional upon infection, a category that was not found among the small number of viable enhancer detector strains that we screened for this phenotype. A gene similar to this category has been found, however, through the identification of the *immune deficiency* (*imd*) mutation (LEMAITRE *et al.* 1995a). *imd* has reduced viability upon bacterial infection and reduced induction of antibacterial protein genes. *imd* has also confirmed that there are at least two immune response pathways, as the gene for the antifungal protein drosomycin is still induced by infection in *imd* (LEMAITRE *et al.* 1995a). The relationship between induction of the immune response and tumor formation is currently unclear and complex. For example, *Toll*, which has Interleukin-1 receptor homology, may participate in regulating induction of the humoral response through *Dif* (IP *et al.* 1993), and *Toll* mutants also have a low percentage of melanotic tumors (GERTULA *et al.* 1988). Also, dorsal moves into the nucleus during an immune response, yet *dorsal* mutants have normal antibacterial protein induction (LEMAITRE *et al.* 1995b). For *Thor*, P-element mobilization combined with molecular localization of the locus can be used to ascertain production of a null mutation and then the role of this gene in viability, response to infection and tumor formation can be directly tested.

The novel approach of identifying mutations with a melanotic tumor phenotype based only on selection of tissue specificity and/or infection inducibility of the reporter gene was successful for tissue specific selection and has identified the genes *wizard* and *dappled*. The lethality of both *wizard* and $dappled^{l(3)l}$ is early in larval development, and thus would have gone undetected in previous screens, which have selected directly for a tumor formation phenotype in late larvae and pupae (*e.g.*, GATEFF 1978; WATSON *et al.* 1991; TÖRÖK *et al.* 1993). The viability of $dappled^{ML8}$ is not unusual for a melanotic tumor mutation, but the combination with 100% tumor formation rather than the typical variable and lower levels makes this mutation a unique practical tool for

isolation of new genes that suppress or enhance melanotic tumors. In general, melanotic tumors are postulated to be a reaction to abnormal development (RIZKI and RIZKI 1980), and this has been extended to the proposal that all melanotic tumor mutants can be categorized as belonging to one of two classes: class 1—melanotic tumors associated with apparently normal immune systems that are responding to abnormal tissues, and class 2—melanotic tumors associated with obvious defects of the immune system's lymph glands and hemocytes (WATSON *et al.* 1991). Since the lymph glands are normal in *dappled*$^{MLB}$ and *dappled*$^{EJI}$, these are class 1 mutations. *wizard* has an expression pattern in lymph glands, oenocytes and head. *wizard* may thus be a class 1 or 2 mutation and requires further analysis to distinguish between the two possibilities. Some class 2 tumor formation genes have been molecularly characterized (WATSON *et al.* 1992; KONRAD *et al.* 1994; HARRISON *et al.* 1995; LUO *et al.* 1995; TÖRÖK *et al.* 1995). Molecular characterization of class 1 tumor formation genes is lacking, and we are cloning *dappled* also to provide molecular information about this category (C.-Y. WU and D. KIMBRELL, unpublished results).

In conclusion, we have identified six novel genes that can be used in the long-term goal of a molecular genetic dissection of immunity and tumor formation. Many more genes than these and previously identified genes are needed for a genetic dissection, and also for providing more of the information that is required for a complete basic description of the relevant systems and processes involved. More than 20 years after the pioneering experiments by BOMAN *et al.* (1972) on humoral immunity and GATEFF (1978) on tumor mutations, the power of Drosophila genetics is still in the early stages of what it can offer in unravelling immunity, tumor formation and their overlap, and in providing a model system for understanding these fundamental processes shared with humans and other organisms.

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