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Drosophila Immunity: Analysis of Larval Hemocytes by *P*-Element-Mediated Enhancer Trap

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ABSTRACT

Our aim was to identify new genes involved in the cellular aspects of defense mechanisms of Drosophila, as well as in melanotic tumor formation processes that are linked to blood cell disregulation. We have screened 1341 enhancer detector fly lines for expression of the *lacZ* reporter gene in larval hemocytes at the end of the third instar. We have selected 21 lines in which we observed a reproducible *lacZ* expression in blood cells. These lines were classified according to the subsets of hemocytes in which *lacZ* was expressed, and we identified five lines that can be used as lamellocyte markers. Three lines were selected for further analysis. The first exhibited strong *lacZ* expression in all lamellocytes. The second expressed *lacZ* in plasmatocytes and lamellocytes, and exhibited a melanotic tumor phenotype in larvae homozygous for the insertion. A third line showed a striking insertion-linked phenotype of melanized lymph glands (the hematopoietic organ), which resulted in the total absence of circulating hemocytes in the mutant larvae. We anticipate that this mutation, which we named *domino,* will prove a useful tool in the analysis of the role of hemocytes during the various aspects of immune response and melanotic tumor formation.

INSECTS are particularly resistant to infections by microorganisms. Their defense reactions rely on both cellular and humoral mechanisms (reviews in HULTMARK 1993; BOMAN 1995; HOFFMANN 1995; HOFFMANN *et al.* 1996). The humoral facet involves the activation of proteolytic cascades, leading to melanization and coagulation and the rapid synthesis of antimicrobial peptides that are released into the hemolymph. A number of these peptides have been isolated from various insect orders (reviews in COCIANCICH *et al.* 1994; BOMAN 1995), and the signaling cascades triggering their synthesis in the fat body, the functional analogue of the mammalian liver, have recently been analysed in Drosophila (LEMAITRE *et al.* 1996). Interestingly, many features of the humoral immune response in insects are reminiscent of the mammalian acute phase response (reviews in HULTMARK 1993; HOFFMANN 1995). The cellular response includes phagocytosis and encapsulation of intruders by the blood cells (reviews in GUPTA 1979; RATCLIFFE 1993). In contrast to the humoral response, the cellular response has been poorly investigated at the level of the molecular mechanisms.

In Drosophila, the role of hemocytes in defense reactions has been documented in larval stages. Although they participate in the synthesis of antibacterial peptides (SAMAKOVLIS *et al.* 1990; MEISTER *et al.* 1994), their major role in the host defense is the phagocytosis of microorganisms and encapsulation of larger intruders such as eggs of parasitic wasps (reviews in RIZKI and RIZKI 1984; NAPPI and VASS 1993).

In Drosophila third instar larvae, hemocytes are derived from the lymph glands that are paired organs associated with the anterior region of the dorsal vessel (DEMEREC 1950; SHRESTHA and GATEFF 1982). Drosophila hemocytes are classically divided into three subtypes: crystal cells, plasmatocytes and lamellocytes (reviewed in RIZKI and RIZKI 1984). Crystal cells account for 5–10% of the blood cell population and are characterized by prominent cytoplasmic paracrystalline inclusions. They are believed to contain the enzymes and the substrate of the prophenoloxidase cascade that is responsible for defense-related melanization processes. Plasmatocytes are small rounded cells with phagocytic capacity; they form the majority of the blood cell population. It was proposed that, at the beginning of pupal life, they differentiate into large flattened lamellocytes. These cells are occasionally observed at earlier stages in defense reactions, namely when they form the walls of capsules enclosing foreign bodies. Lamellocytes are also associated with the formation of melanotic pseudo-tumors (review in SPARROW 1978).

Our information on hemocyte lineages and functions remains fragmentary, largely through the lack of genetic markers. This has led us to undertake the screen of *P-lacZ* enhancer trap lines to identify fly lines with transgene expression in subsets of blood cells. The rationale was to obtain specific markers associated with various cell types. We were particularly interested in markers for lamellocytes, as these cells are involved in

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self/non-self recognition leading to encapsulation and melanotic tumor formation. Moreover, given that these cells massively differentiate at metamorphosis, they are presumably also associated with tissue remodeling.

We have devoted particular attention to insertion-linked phenotypes that affect blood cells and have identified a novel *P*-element mutation that results in the total absence of circulating hemocytes in larvae.

MATERIALS AND METHODS

Drosophila stocks: 491-*lacZ* enhancer detector stocks were from the collections of TÖRÖK *et al.* (1993) and 636 from the Indiana Drosophila Stock Center [Berkeley Drosophila Genome Project (BDGP) stocks]. One hundred seventy-three enhancer trap lines were generated in this laboratory by *P*-element mutagenesis following the crossing scheme of BIER *et al.* (1989) with a P-lacW ammunition stock. Forty-one P-lacW fly lines that express *lacZ* in the adult fat body and/or ovaries were provided by Dr. J. A. LEPESANT (Institut Jacques Monod, Paris). These enhancer detector elements all contain a fusion of the *lacZ* gene to exon I of the *P* transposase gene and either the $rosy^+$ or $white^+$ genes as markers (BELLEN *et al.* 1989; BIER *et al.* 1989). In the lines that were further analyzed, P-*lacZ* insertions on the second chromosome were balanced with a $CyO\ y^+$ chromosome in a *y,w* context on the *X* chromosome. Homozygous larvae could thus be distinguished by their yellowish mouth parts. Third chromosome insertions were balanced by the TM6B balancer and homozygous larvae were thus distinguished from their siblings as $Tubby^+$. *CyO, elav-lacZ* was used as an embryonic marker and was obtained from the Bloomington Stock Center.

The transgenic p50-*lacZ*:6 line (GOVIND 1995) was used as a positive control in the study of hemocyte stainings. In this line, the fusion gene contains the *hsp83* promoter upstream of the Rel-homology region of the murine p50 protein stabilized by the *lacZ* C-terminus. The larval hemocytes in this strain exhibit strong constitutive *lacZ* expression.

The lethal enhancer trap line esg^{P3} is a strong allele of the *escargot* gene (HAYASHI *et al.* 1993) and expresses *lacZ* in the neuroblasts and imaginal discs in larvae. It was used as a marker for these structures in *domino* mutants.

$Toll^{10b}$ is a dominant gain-of-function ventralizing allele of *Toll* caused by a single amino acid change (SCHNEIDER *et al.* 1991). $Toll^{10b}/+$ females produce strongly ventralized embryos. In addition, an early differentiation of plasmatocytes into lamellocytes together with a melanotic tumor phenotype are observed in larvae carrying this mutation (GERTULLA *et al.* 1988; LEMAITRE *et al.* 1995). The $Toll^{10b}$ mutation also induces a constitutive activation of the drosomycin gene that encodes an antifungal peptide (LEMAITRE *et al.* 1996).

$hop^{Tum\text{-}l}$ is a thermosensitive dominant gain-of-function allele of *hopscotch* caused by a single amino-acid change (HARRISON *et al.* 1995; LUO *et al.* 1995). $hop^{Tum\text{-}l}$ mutants are lethal at 29° and exhibit an overproliferation of plasmatocytes at all culture temperatures, with a substantial portion of these cells that prematurely differentiate into lamellocytes (HANRATTY and DEAROLF 1993; LUO *et al.* 1995).

Black cells (*Bc;* RIZKI *et al.* 1980) is a dominant mutation that is characterized by the presence in heterozygous mutants of circulating melanized crystal cells. When hemolymph is withdrawn from *Bc* larvae, the crystal cells do not disrupt. Homozygous larvae and adults have no phenoloxidase activity in the cell-free hemolymph and fail to darken after injury (RIZKI *et al.* 1980). Deficiency stocks and markers are described in LINDSLEY and ZIMM (1992). Experiments and crosses were performed at 25°.

Septic injury: Third instar larvae were pricked with a sodium nitrite sharpened tungsten needle previously dipped every time into a concentrated bacterial culture of *Escherichia coli* and *Micrococcus luteus* (OD of the bacterial pellet estimated to ~100).

Histochemical detection of β-galactosidase activity: Dissected larvae were either stained directly for β-galactosidase activity or analyzed after fixation (in the case of the 491 lines from TÖRÖK *et al.* 1993) in 1% glutaraldehyde in phosphate-buffered saline (PBS) pH 7.5, for 5 min at 4°. Staining was in 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 3.5 mM $K_4Fe(CN)_6$, 3.5 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$, 150 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 25% Ficoll-400 overnight at room temperature (HIROMI *et al.* 1985).

Hemocytes were prepared as follows: wandering third instar larvae were washed in distilled water, dried, then punctured posteriorly and gently squeezed to deposit a droplet of hemolymph (<1 μl) on a glass coverslip. After 5 min drying, the preparations were fixed for 30 sec in a 0.5% glutaraldehyde/PBS solution and treated as described above. Staining was overnight at 37°. Hemocyte preparations were mounted in glycerol. A positive control experiment was systematically run with each experimental series, using a transgenic strain in which hemocytes express *lacZ* (GOVIND 1995).

Transmission electronic microscopy: Lymph glands were dissected in PBS, fixed in 0.1 M sodium phosphate buffer (pH 7.3)/1.5% glutaraldehyde/1.5% formaldehyde for 1 hr at 4°, postfixed with osmium tetroxide, counterstained with lead citrate and uranyl acetate, embedded in plastic, and sectioned for electron microscopy.

Histology: First or third instar larvae were fixed in Carnoy's fixative and either stained as whole mounts with toluidin/eosin, or embedded in paraffin. Subsequent histological sections were stained in Hansen's haematoxylin/erythrosin.

Immunohistochemistry: Embryos were collected, dechorionated, fixed in a 1:1 mixture of heptane and 4% formaldehyde in PIPES buffer, methanol devitellinized, and treated with 0.3% H_2O_2 in methanol for 20 min. Embryos were rehydrated and blocked in 0.2% Tween-20, 2% serum in PBS for 1–3 hr, and then incubated overnight with primary antibody at a dilution of 1:200. Mouse anti-β-galactosidase was from Tebu and affinity-purified rabbit anti-croquemort antibody (FRANC *et al.* 1996) was kindly provided by JEAN-LUC DIMARCQ (Strasbourg). Secondary antibodies were horse-radish peroxidase-coupled anti-rabbit Ig (Amersham) and an anti-mouse IgG Elite ABC kit (Vectastain). *domino* mutant embryos were identified by the absence of *elav-lacZ* expression.

RESULTS

We have screened 1341 P-*lacZ* fly lines for expression of the reporter gene in larval tissues at the end of the third larval instar (wandering stage); 1111 fly lines were lethal, 45 were male-sterile and 185 nonlethal autosomal insertions. The lethal lines consisted of the following: (1) 591 enhancer detector stocks from the Indiana Drosophila Stock Center (BDGP stocks) with insertions on the second and third chromosome, (2) 491 stocks from the collection of TÖRÖK *et al.* (1993) that were late larval or pupal lethals due to second chromosome insertions, (3) 29 autosomal insertions generated in this laboratory by *P*-element mutagenesis using a P-lacW ammunition stock. The male sterile lines were BDGP stocks, and the viable lines consisted of 144 autosomal insertions that we generated and 41 lines that had been

TABLE 1

Expression of the P-*lacZ* reporter gene at the end of the third larval instar

No. of tissues with *lacZ* expression	No. of lines	Percentage
0	346	25.8
1 (ID)	324 (188)	24.2 (14.0)
2	259	19.3
3	173	12.9
4	87	6.5
5	73	5.4
6	36	2.7
7	20	1.5
8	10	0.7
9	7	0.5
10	5	0.4
11	1	0.07

Expression of the P-*lacZ* reporter gene at the end of the third larval instar. The percentages are given relative to the 1341 different fly lines that were screened. The numbers in parentheses are for imaginal discs (ID) as the unique *lacZ* expressing tissue. The *lacZ* staining patterns of the BDGP stocks are available to the scientific community on the FlyBase databank.

TABLE 2

Tissues expressing the P-*lacZ* reporter gene at the end of the third larval instar

Tissue with *lacZ* expression	No. of lines	Percentage of all lines	Percentage of staining lines
Nonstaining	346	25.8	
Ant im. discs	478–488	35.6–36.4	48.0–49.0
Foregut	169	12.6	17.0
Midgut	422–426	31.5–31.8[a]	42.4–42.8[a]
Hindgut	240–241	18.0	24.1
Gut (total)	519	38.7	52.2
Saliv. glands	317–318	23.6–23.7	31.9–32.0
Gonadal discs	302–307	22.5–22.9	30.4–30.9
Brain	255–261	19.0–19.5	25.6–26.2
Malpigh. tub.	253	18.9	25.4
Fat body	202–203	15.1	20.3
Ring gland	132–138	9.8–10.3	13.3–13.9
Trachaea	91–92	6.8–6.9	9.1–9.2
Integument	83–86	6.2–6.4	8.3–8.6
Oenocytes	45–48	3.4–3.6	4.5–4.8
Lymph glands	29–43	2.2–3.2[b]	2.9–4.3[b]
Hemocytes	21	1.6	2.1

Tissues expressing the P-*lacZ* reporter gene at the end of the third larval instar. The percentages are given relative to the 1341 different lines or to the 995 lines in which a *lacZ* expression was observed in larvae. When two numbers are given, some lines show weak staining close to background. Ant. im. discs, anterior imaginal discs.

[a] Possibly overestimated due to the endogenous β-galactosidase activity in the midgut.

[b] Probably underestimated as lymph gland staining was not easy to score in the case of strong blue coloration in the neighboring brain and imaginal discs.

preselected for *lacZ* expression in adult fat body and/or ovaries. The larvae were pricked with a bacteria-soaked needle, scored for β-galactosidase activity in all tissues and hemocytes 6 hr after challenge, and compared with naive animals. In all lines that were tested, we never observed a *lacZ* expression that was induced by septic injury. We will first describe the global expression patterns of the reporter gene in third instar larvae and then analyze the hemocyte staining lines.

***LacZ* expression pattern in third instar larvae:** The expression patterns of the reporter gene at the end of the third larval instar are summarized in Tables 1 and 2. Among the 1341 lines, 346 exhibited no β-galactosidase activity. Table 1 shows the percentage of lines that stained in one to several (up to 11) different tissues that included brain, imaginal discs, gut, fat body, lymph gland, ring gland, oenocytes, Malpighian tubules, integument (the epidermis, cuticle and muscles that constitute the carcass), trachaea and hemocytes. We found staining in only one tissue in 324 lines, and the majority of these unique stainings were in imaginal discs (188 lines). More frequently, the β-galactosidase activity was seen in several different tissues and we observed a wide range of combinations in the various groups. Table 2 gives the number of lines that stained per given tissue. The most frequent *lacZ* activities were recorded in the gut and in the imaginal discs, both of which exhibited an array of distinct expression patterns, as previously described in related studies (Murakami *et al.* 1994; Goto *et al.* 1995). Frequent β-galactosidase activity was also detected in salivary glands, in the brain and in Malpighian tubules. Only 15% of the tested lines had fat body staining, which is in fact a slight overestimate, as 41 of the fly lines had been previously selected for β-galactosidase activity in the fat body (stocks from J. A. Lepesant, Paris). Finally, we found a reproducible coloration in blood cells in 21 lines (1.6%).

We further analyzed the results in the lines with *lacZ* expression in the two major immunoresponsive tissues, namely the fat body and the hemocytes. Some 200 fly lines expressed the reporter gene in the fat body. As is the case for all larval staining lines in our series, these fat body lines showed frequent concomitant *lacZ* expression in the gut, imaginal discs, salivary glands and Malpighian tubules, followed by the brain (Figure 1A and Table 2).

A similar analysis in the 21 hemocyte-staining lines (Figure 1B, see also Table 4) showed frequently associated staining in the gut (86%), in the imaginal discs (71%) and in the Malpighian tubules (57%). The striking observation here was that a *lacZ* expression was scored in the fat body and in the lymph glands in half of the hemocyte-staining lines. This contrasts with the general pool of staining lines in which only 20% exhibited fat body staining and 3–4% lymph gland staining (Table 2). Ring gland, trachaea and integument *lacZ* expression were also frequently associated with hemo-

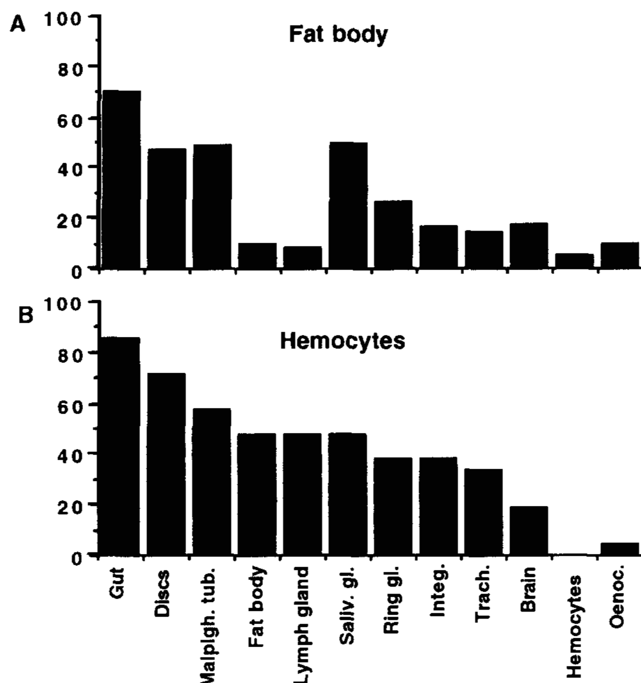

FIGURE 1.—Histograms showing frequency (percentages) at which *lacZ* expression in larval fat body (A) or hemocytes (B) is combined with expression in other tissues (indicated below): gut, imaginal discs, Malpighian tubules, fat body, lymph glands, salivary glands, ring gland, integument, trachaea, brain, hemocytes and oenocytes. Bars representing combination of fat body or hemocytes with itself give the frequency at which expression occurs in these tissues exclusively.

cyte staining, although this co-expression was less marked than between hemocytes and fat body/lymph glands.

Analysis of the hemocyte-staining lines: We have further analyzed *lacZ* expression in larval hemocytes. A detailed analysis of blood cell subtypes was hampered by the fact that even after injury, the hemolymph contained predominantly plasmatocytes. We therefore used a genetic approach to be able to observe lamellocytes or crystal cells. We analyzed the 21 hemocyte-staining lines in mutant backgrounds known to affect blood cells. *Toll*^{10B} and *hopscotch*$^{Tumorous\text{-}lethal}$ (*hop*$^{Tum\text{-}l}$) mutations were used to obtain lamellocytes, and the *Black cells* (*Bc*) mutation for crystal cells.

Toll^{10B} is a dominant gain-of-function mutation of the gene encoding the Toll transmembrane receptor, which is constitutively active (SCHNEIDER *et al.* 1991). The *Toll*^{10B} mutation results, in larvae, in precocious differentiation of plasmatocytes into lamellocytes and in a melanotic tumor phenotype (GERTULLA *et al.* 1993; LEMAITRE *et al.* 1995). *hop*$^{Tum\text{-}l}$ is a dominant mutation of the JAK-kinase hopscotch that is constitutively active in larval hemocytes of the mutants (HANRATTY and DEAROLF 1993; BINARI and PERRIMON 1994; HARRISON *et al.* 1995; LUO *et al.* 1995). *hop*$^{Tum\text{-}l}$ generates a neoplasm of the lymph glands, with concomitant overproliferation of hemocytes and early differentiation into lamellocytes. It also produces a melanotic tumor phenotype. *Bc* mutants are characterized by the presence in the hemocoel of circulating melanized crystal cells (RIZKI *et al.* 1980). When hemolymph is withdrawn from *Bc* larvae, the crystal cells do not disrupt and *lacZ* expression can be monitored in these cells that contain black crystals. As these three mutations are dominant, a single cross is sufficient to obtain larvae with both the mutant phenotype and a copy of the reporter gene.

The analysis of the 21 hemocyte-staining lines in the three mutant backgrounds led us to group them into three classes (Table 3): (1) lines with hemocyte *lacZ* expression restricted to plasmatocytes, (2) lines with expression both in plasmatocytes and in lamellocytes, (3) lines with expression predominantly in lamellocytes.

In the first class comprising five lines, the staining was usually observed in a subfraction of the plasmatocyte population, but not in all cells (Figure 2A), suggesting that each group of stained plasmatocytes corresponds to a defined subpopulation. However, when each of the five lines was crossed with the others, the percentage of stained cells did not increase in the resulting progeny (data not shown). Interestingly, this group includes the line *l(3)05309* carrying an insertion in the *Tenascin*^{m} (*Ten*^{m}) gene that encodes an extracellular matrix protein (BAUMGARTNER *et al.* 1994; LEVINE *et al.* 1994).

The second class is composed of 11 fly lines (Figure 2B). Again in plasmatocytes we never recorded 100% staining, but in some lines all lamellocytes showed β-galactosidase activity (*l(3)j5C2, l(3)j2D1* and *l(3)05203*), particularly in the *hopTum-l* context. In two lines that exhibit a pupal lethality (line *l(3)03463* and *l(3)03550*) we observed a noticeably higher percentage of stained plasmatocytes in larvae homozygous for P-*lacZ* than in heterozygous larvae.

In the third class that comprises five fly lines, the hemocyte β-galactosidase activity was observed predominantly, or only, in lamellocytes (Figure 2, C–E). A small proportion of the plasmatocyte population stained in these lines, suggesting that they were differentiating into lamellocytes.

Both in the plasmatocyte and the lamellocyte staining classes, we observed occasional *lacZ* expression in crystal cells (Table 3 and Figure 2F).

When we looked for β-galactosidase activity in larval lymph glands in these 21 fly lines, we found that the hematopoietic organ expressed *lacZ* only in lines that exhibited significant plasmatocyte staining (Table 4 and Figure 3A). The lymph gland did not stain in the third class of fly lines with mainly lamellocyte staining.

It is worth noting that we did not find an exclusive hemocyte *lacZ* expression in the course of the screen. In the 21 hemocyte-staining lines analyzed, we observed that at least two to five other tissues expressed the reporter gene (Table 4). Line *ms(2)05158*, which belongs

TABLE 3

Classification of the hemocyte-staining P-*lacZ* lines

Stock name	Cytological location	Original context Plasmatocyte staining							*hop Tum-l* context Lamellocyte staining					*Toll 10B* context Lamellocyte staining					*Bc* context Crystal cell staining					Characteristics
		0%	<1%	1–10%	>10% <50%	>50% <90%	>90%	nL/nT	0%	<10%	>10% <90%	>90%	nL/nT	0%	<10%	>10% <90%	>90%	nL/nT	0%	<10%	>10% <90%	>90%	nL/nT	
lacZ expression in plasmatocytes only																								
l(2)00642	47A11-12					++		20/3	NS				8/2	NS				3/2		(+)			6/1	
l(3)02414	85F12-13			+				30/4	NS				2/1	NS				6/1	NS				5/1	l(P); h = H
l(3)05309	79E1-2					+		20/3	NS				5/2	NS				3/1			+		5/1	*Tenascin m* allele
l(3)10052	68A1-2		←		+var		→	30/4	NS				4/2	NS				5/1	NS				5/1	semilethal; h = H
ms(3)07735	82C?		++					50/6	NS				6/2	NS				11/2	NS				5/1	male sterile; h = H
lacZ expression in plasmatocytes and lamellocytes																								
l(2)03350	21B4-6			+				30/4			(+)		5/1		(+)			7/2			(+)		9/1	
l(3)00865	100A1-2			+				20/3		+			8/2		(+)			6/2		+			5/1	
l(3)01235	99A5-6						+	20/3			(+)		6/2		((+))			11/2			+		5/1	
l(3)03463	87D7-9			+h		+H		30/4			(+)		19/4			(+)		31/4	NS				10/2	l(P); h < H
l(3)03550	88E8-9			+h			++H	30/4			+		22/5		(+)			28/6		(+)			5/1	l(P); h < H; melanotic tumors
ms(2)05158	28A				(+)			25/3			+		16/3		(+)			18/5		+			6/1	male sterile; h = H
l(3)j5C8	72D1-2				+			25/4		(+)			18/5	NS				24/5		(+)			10/2	
l(2)10403	52E5-6		←		+var		→	40/5			(+)		4/1		(+)			7/1			+		6/1	
l(3)j5C2	63B7-8				++			20/3				+	4/1				(+)	5/1			+		5/1	
l(3)j2D1	93C1-3			←	+var	→		40/5				+	4/1				+	3/1			+		5/1	
l(3)05203	89B12-13			←	+var		→	45/6				+	4/2			+		5/1			+		5/1	few escapers; h = H
lacZ expression mainly in lamellocytes																								
l(2)01272	30C1-2		++					40/6				+	8/2			+		8/2	NS				5/1	
l(3)06946	62E6-7			(+)				30/4				+	8/2				+	12/3		+			5/1	
l(3)03349	66E6-7		+					30/4				++	11/2				++	25/5		+			21/3	semilethal; l(P)
l(2)27/7	Chromosome 2		←	(+)var	→			25/3				+	7/1				+	10/1			+		10/1	l(P); h = H
l(2)113/28	34A5-6	NS						25/3				+	4/1				+	8/1		+			10/1	l(P); h = H

Classification of the hemocyte-staining P-*lacZ* lines. The hemocytes that express β-galactosidase are given in percentage classes for each subtype: plasmatocytes in wild-type context, lamellocytes in hop^{Tum-l} or $Toll^{10B}$ contexts, crystal cells in *Bc* context. The last column of each series indicates the total number of larvae that were tested (nL) in a given number of tests (nT). The staining intensities are reported as follows: ((+)), very weak; (+), weak; +, intermediate; ++, strong. h stands for larvae heterozygous and H for larvae homozygous for the P-*lacZ* insertion. h = H means that the staining intensity in plasmatocytes was equivalent for larvae heterozygous and homozygous for the P-*lacZ*, h < H means that it was significantly stronger in homozygotes (the P-*lacZ* insertion was always heterozygous in $Toll^{10B}$, hop^{Tum-l} and *Bc* contexts). NS, nonstaining; var, variable percentages in different individuals; l(P), pupal lethality.

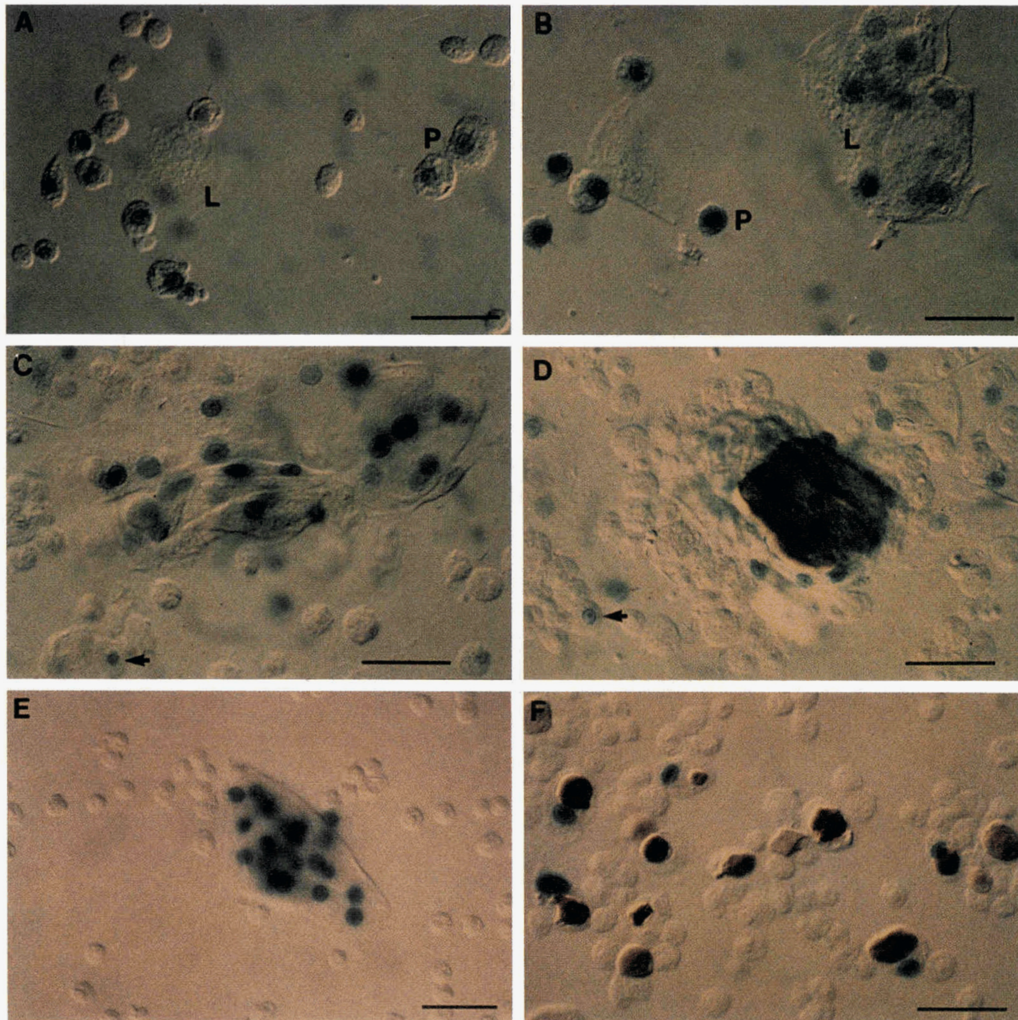

FIGURE 2.—*lacZ* expression in larval hemocytes. (A Staining in plasmatocytes (P) in line *l(3)05309* (insertion in the *Ten*^{m} gene). Note the presence of a lamellocyte (L) that does not express *lacZ*. (B) Plasmatocytes and lamellocytes in line *l(3)01235* in *hop*$^{Tum\text{-}l}$ context. The staining is strong in plasmatocytes and weaker in lamellocytes. (C and D) Lamellocyte-staining in line *l(3)03349* in *hop*$^{Tum\text{-}l}$ (C) or *Toll*^{10B} (D) contexts. Some plasmatocytes do also express *lacZ* (arrows). Note the presence, in *Toll*^{10B}, of a free floating melanotic tumor surrounded by lamellocytes. (E) Exclusive lamellocyte staining in line *l(2)113/28* in *Toll*^{10B} context. (F) *lacZ* expression in some crystal cells in line *l(3)05203* in *Bc* context. Bars, 40 μm.

to the second class of hemocyte-staining lines, represents an extreme case as it showed an ubiquitous β-galactosidase activity in all tissues at the end of the third larval instar.

Seventeen of the 21 fly lines corresponded to lethal insertions, two lines (*l(3)10052* and *l(3)03349*) were semi-lethal and two were male-sterile (*ms(2)05158* and *ms(3)07735*). Of the 21 lines, six exhibited a pupal lethality or semi-lethality.

In line *l(3)03550* where a high percentage of hemocytes and lymph glands (Figure 3A) strongly expressed *lacZ* (together with imaginal discs, midgut and to a lesser extent Malpighian tubules), we observed in homozygous larvae a high penetrance of a melanotic tumor phenotype: third instar larvae contained circulating melanotic capsules (Figure 4A) and died as early pupae. This is the only line out of the 21 hemocyte-staining lines in which we observed this phenotype. We found several other lines with melanotic tumor phenotypes in the process of screening, but did not retain them as they did not show hemocyte or lymph gland *lacZ* expression.

Characterization of a mutant devoid of circulating hemocytes: In the screen for immune-related phenotypes, we identified a novel mutation on the second chromosome (*l(2)81/8*) in which homozygous larvae were totally devoid of circulating hemocytes. When placed into favorable conditions at the beginning of the third instar (*i.e.*, separated from heterozygous larvae with wild-type phenotype), these larvae had a prolonged third instar, up to 10 days at 20°. They exhibited a striking phenotype of melanized lymph glands (Figure 4B), which became apparent during the second larval instar when a few black dots developed in the anterior lobes of the hematopoietic organ. During the third instar, this blackening progressively invaded the whole lobes and later extended to the posterior lobes. The melanized lymph gland lobes eventually detached from the dorsal vessel and in aging third instar larvae, black debris was seen floating along the dorsal vessel (not shown). The mutant larvae were able to pupariate but died at stage 5–6 hr after puparium formation (according to pupal stages as determined by BAINBRIDGE and BOWNES 1981).

The ultrastructural observation of lymph glands showed that in mutant larvae, the prohemocytes in the

TABLE 4

Larval *lacZ* expression in hemocyte-staining lines

Stock name	Cytologic location	Lymph gland staining	β-galactosidase staining in larval tissues
l(2)00642	47A11-12	+	ID +; FB +; Integ +
l(3)02414	85F12-13		ID (+); HG +
l(3)05309	79E1-2		ID +; MG (+)
l(3)10052	68A1-2		Br +; ID (+); HG (+); Integ +
ms(3)07735	82C?		ID (+); Gut + (pattern); MT (+); SG ++; Integ +; Tra ++
			Lines with *lacZ* expression in plasmatocytes and lamellocytes
l(2)03350	21B4-6	+	Br (+); ID ++; Gut ++; MT +; SG ++; FB +
l(3)00865	100A1-2	(+)	Br (+)
l(3)01235	99A5-6	+	ID ++; GD ++
l(3)03463	87D7-9	+	ID ++; AG&MG +; MT +; FB +
l(3)03550	88E8-9	++	ID +; MG + (pattern); MT (+); GD +
ms(2)05158	28A	+	Br +; RG +; ID ++; Gut +; MT ++; SG ++; FB +; GD +, Integ +; Tra +
l(3)j5C8	72D1-2	(+)	RG +; ID +; Gut +; MT +; SG ++; FB +; Integ (+); Tra (+)
l(2)10403	52E5-6		RG +; Gut +; SG +; FB +
l(3)j5C2	63B7-8	+	RG ++; ID (+); Gut ++; MT ++; SG ++; FB ++; Tra +
l(3)j2D1	93C1-3	++	Gut +; MT (+); SG (+); FB (+)
l(3)05203	89B12-13		PV +; MG +; HG (+); MT (+); FB +; Tra (+)
			Lines with *lacZ* expression mainly in lamellocytes
l(2)01272	30C1-2		Gut +; MT +; SG +; Integ +; Tra +
l(3)06946	62E6-7		RG +; ID (+); Gut +
l(3)03349	66E6-7		RG +; ID +; MG (+)
l(2)27/7	Chromosome *2*		RG (+); Gut ++; MT +; SG ++; GD (+); Integ +
l(2)113/28	34A5-6		RG +; HG ++; MT ++; SG ++; FB +; Oeno (+); Integ (+); Tra +

Larval *lacZ* expression in hemocyte-staining lines. The various tissues in which we observed a *lacZ* expression in late third instar larvae are given as follows: AG, anterior gut; Br, brain; FB, fat body; GD, gonadal disc; HG, hindgut; ID, anterior imaginal discs; Integ, integument (cuticule + epidermis + muscle layers); MG, midgut; MT, Malpighian tubules; Oeno, oenocytes; RG, ring gland, SG, salivary gland; Tra, trachea; and independently in the third column for the lymph glands. Staining intensities are as in Table 3. Cytological locations are as provided by the Bloomington Stock Center or by B. Mechler.

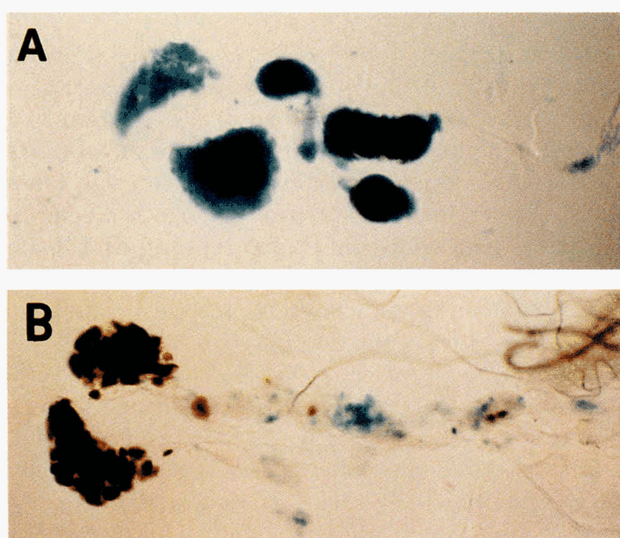

Figure 3.—*lacZ* expression in lymph glands of line *l(3)03550* (A) and *domino* (*l(2)81/8*) (B) third instar larvae. Note the intense melanization in the first lobe of *domino* lymph glands.

hematopoietic organ were considerably larger than in wild type (Figure 5). Whereas wild-type lymph glands of third instar larvae contain predominantly clonal clusters of differentiating prohemocytes, the lymph glands of this mutant were filled with both necrotic, melanized cells and cells packed with heterogeneous inclusions indicative of strong resorptive processes (*e.g.*, multivesicular bodies, autophagosomes with multilamellar whirls, Lockshin and Beaulaton 1979). The mutant glands were devoid of differentiating prohemocytes, which explains the absence of circulating hemocytes. Because of the very striking lymph gland phenotype that results in mutant larvae with two black dots visible on the anterior half, we named the mutation *domino.*

We looked for the presence of hemocytes in *domino* mutants at earlier stages of development. Embryonic hemocytes derive from anterior mesoderm and can be first identified in the head at stage 10–11. Then they disperse along several migratory paths throughout the embryo and are responsible for the phagocytosis of apoptotic cells (Tepass *et al.* 1994). We used an antibody against Croquemort, a CD36 homologue that was shown in embryos to be exclusively expressed in hemo-

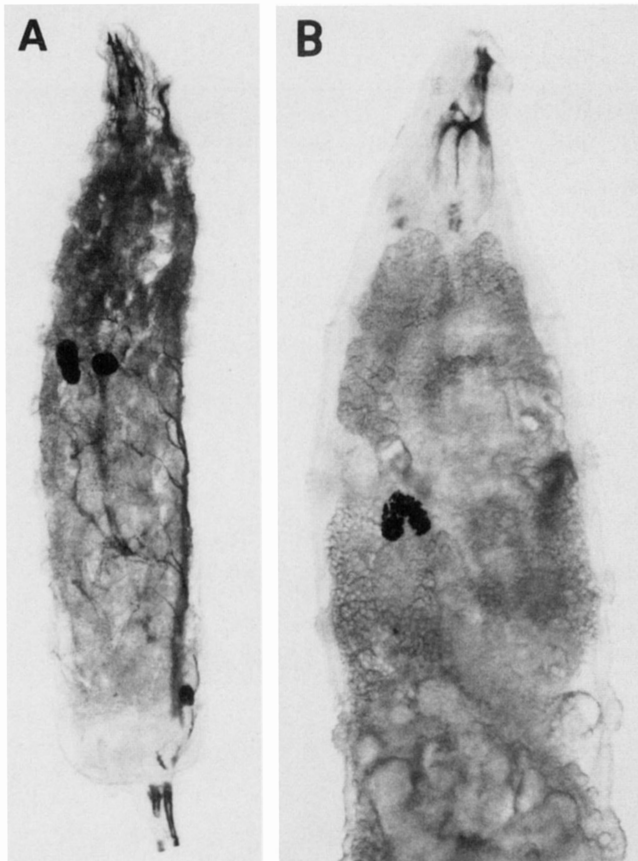

FIGURE 4.—Phenotypes of two mutant larvae. (A) Melanotic tumors in a homozygous *l(3)03550* third instar larva. (B) Melanized lymph glands in a *domino* (*l(2)81/8*) third instar larva.

cytes from stage 13 onward and to participate in the recognition of apoptotic cells (FRANC *et al.* 1996). In *domino* mutants, the distribution of Croquemort-labeled macrophages was similar to that of wild-type embryos (Figure 6A), indicating that this mutation does not affect embryonic hemocytes. The analysis of later stages was achieved by whole-mount histochemical staining of larvae and direct examination of the inner body wall for the presence of nested hemocyte clusters. In first instar larvae, hemocytes were still observed in *domino* mutants, but their number was reduced compared to wild type and, strikingly, they were considerably oversized with an abnormal aspect (Figure 6, B and C). Later, their number decreased and they could hardly be found in the hemocoel of third instar larvae.

In addition to their disregulated hematopoiesis, the mutant larvae appeared devoid of imaginal discs (Figure 6, D and E), of imaginal rings and histoblasts in the gut (not shown), and the size of the brain was significantly reduced (see Figure 6E). To investigate this phenotype, we used esg^{P3}, a *P-lacZ* insertion in the *escargot* gene, as a marker for larval imaginal discs and neuroblasts (HAYASHI *et al.* 1993). We recombined esg^{P3} and *domino* on the second chromosome, and the esg^{P3} activity was scored in larvae heterozygous for the marker and homozygous for *domino*. In third instar larvae, the esg^{P3} marker showed that brain neuroblasts are still present in *domino* mutants, but their domain in the optic lobes is markedly reduced (Figure 6, F and G). The esg^{P3} *lacZ* expression revealed the existence in many larvae of residual imaginal discs associated with the brain, which indicates that they are not totally absent, but can subsist as small clusters of large cells (Figure 6H). Finally, the genital discs also exhibited a reduced size in *domino* larvae.

As the *domino* mutation results from the insertion of a P-*lacZ* element, we scored *lacZ* expression in embryos and third instar larvae in this stock. No β-galactosidase was detected in embryos; in larvae (Table 5), staining was observed in lymph glands (in the cells that were not yet melanized, see Figure 3B) and was strong in the gut of homozygous animals. Weak staining was observed in the imaginal discs, ring gland, salivary glands, gut and lymph glands of heterozygous larvae, but not in hemocytes.

The observed phenotype resulted from a mutation on the second chromosome. By crosses with deficiency stocks, we established that stock Df(2R)AA21, which uncovers region 56F9-17 to 57D11-12, and stock Df(2R)Pu-D17, which uncovers 57B4 to 58B, do not complement the mutation. We therefore could map it to the region comprised between 57B4 and 57D11-12. Precise *P*-element excision reverted the phenotype demonstrating that the mutation resulted from the insertion of a *P* element. We further localized this insertion, by hybridization of a *P*-specific probe to polytene chromosomes, to region 57E1-2 (data not shown), which borders the region as mapped by the deficiencies.

DISCUSSION

The primary aim of this screen was the identification of enhancer trap fly lines expressing the reporter gene in hemocytes. We have described here 21 fly lines with blood cell-staining at the end of the third larval instar, and two insertion-linked phenotypes related to blood cell disregulation. We anticipate that the characterization of the genes adjacent to the transposons will contribute to our understanding of the functions of larval hemocytes, namely in the immune response or in tissue remodelling during metamorphosis.

In addition, we provide the *lacZ* staining patterns in wandering larvae for 1341 enhancer-trap lines. Whereas similar analyses have already been published for embryos (BELLEN *et al.* 1989; BIER *et al.* 1989; HARTENSTEIN and JAN 1992), this is the first extensive report for this developmental stage. The proportion of nonstaining lines in our study was in keeping with that reported in embryos (BELLEN *et al.* 1989; BIER *et al.* 1989) and we observed a similar high frequency of *lacZ* expression in the gut and in the brain. The major

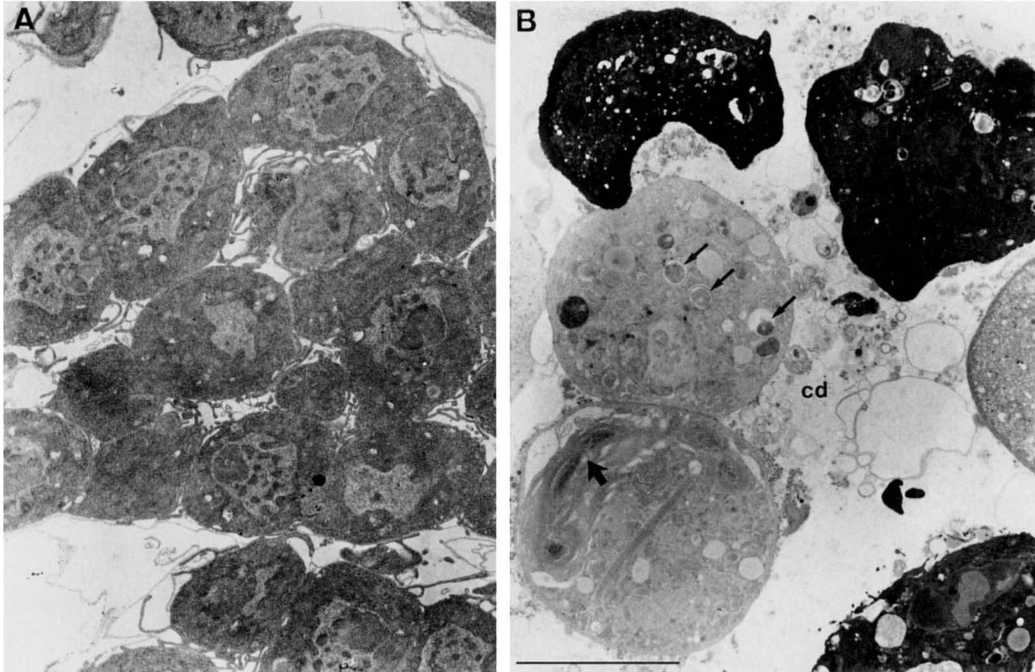

FIGURE 5.—Transmission electron micrograph of a wild-type Oregon[R] (A) and a homozygous *domino* (*l(2)81/8*) lymph gland (B). In the mutant lymph gland, two types of cells are visible: dead cells that are totally melanized and round cells containing either heterogenous bodies of resorptive nature (thin arrows) or autophagic vacuoles with membrane whirls (thick arrow). Between the cells, there is an accumulation of cell debris (cd) resulting from the disruption of the plasma membrane of dead cells. Bar, 10 μm (same scale for both micrographs).

difference between embryonic and larval patterns is that in embryos the tissues that most frequently express *lacZ* are the central nervous system followed by gut, whereas in wandering stage larvae, the gut and imaginal discs prevail.

We have presented in this article a tentative classification of the hemocyte-staining lines in relation to the subsets of blood cells that express the *lacZ* reporter and have described three groups with either of the following: (1) plasmatocyte staining, (2) plasmatocyte and lamellocyte staining and (3) predominant lamellocyte staining. A major result of this classification of blood cell staining lines is that we have identified five enhancer trap lines that can be used as markers for larval lamellocytes. Only in lamellocytes were we able to reproducibly observe 100% staining in all tested larvae in six lines (the five lamellocyte-marker lines plus a lamellocyte and plasmatocyte staining line). Plasmatocyte expression was never observed in all cells, which suggests a heterogeneity in this population. We could not however find evidence of distinct, exclusive, subgroups by crossing fly lines to one another. It seems that the staining patterns in plasmatocytes are more related to the level of activity of equivalent cells rather than to different cell types. The data on crystal cells cannot be correlated with staining or absence of staining in other blood cells, but this is probably due to a bias in the initial screen that was performed mainly on plasmatocytes; we therefore could not expect to find specific crystal cell markers.

Fifty percent of the hemocyte-staining lines also expressed *lacZ* in the fat body, which indicates that many transposon-containing genes that are activated in blood cells are also expressed in fat body cells. This is known to be the case, at least at embryonic stages, for genes encoding proteins of the extracellular matrix that have already been characterized in Drosophila: *Tenascin*^{m} (BAUMGARTNER *et al.* 1994), peroxidasin, laminins, collagen IV, glutactin, tiggrin and papilin (reviewed in FESSLER *et al.* 1994). It is significant in this respect that one of the hemocyte-staining lines which we identified corresponds to an insertion in the *Tenascin*^{m} gene (BAUMGARTNER *et al.* 1994). The expression of *Ten*^{m} has been described in embryonic hemocytes and our results indicate that this protein is most probably synthesized in hemocytes at later stages of development. It is indeed likely that larval hemocytes participate in the synthesis of extracellular matrix as do embryonic hemocytes. Extracellular matrix molecules are components of the basement membranes and accumulate in the intercellular spaces; together with their cognate cell surface receptors (such as integrins), they regulate the relations between the cells and their microenvironment. These interactions are essential not only in developmental processes, but were also shown in vertebrates to be involved in immune-related mechanisms (reviews in SPRINGER 1990; HYNES 1992). In Drosophila, peroxidasin has been proposed to contribute to immune mechanisms as it shares a peroxidase domain with human oxidative defense proteins (NELSSON *et al.* 1994). It is remarkable that, except for

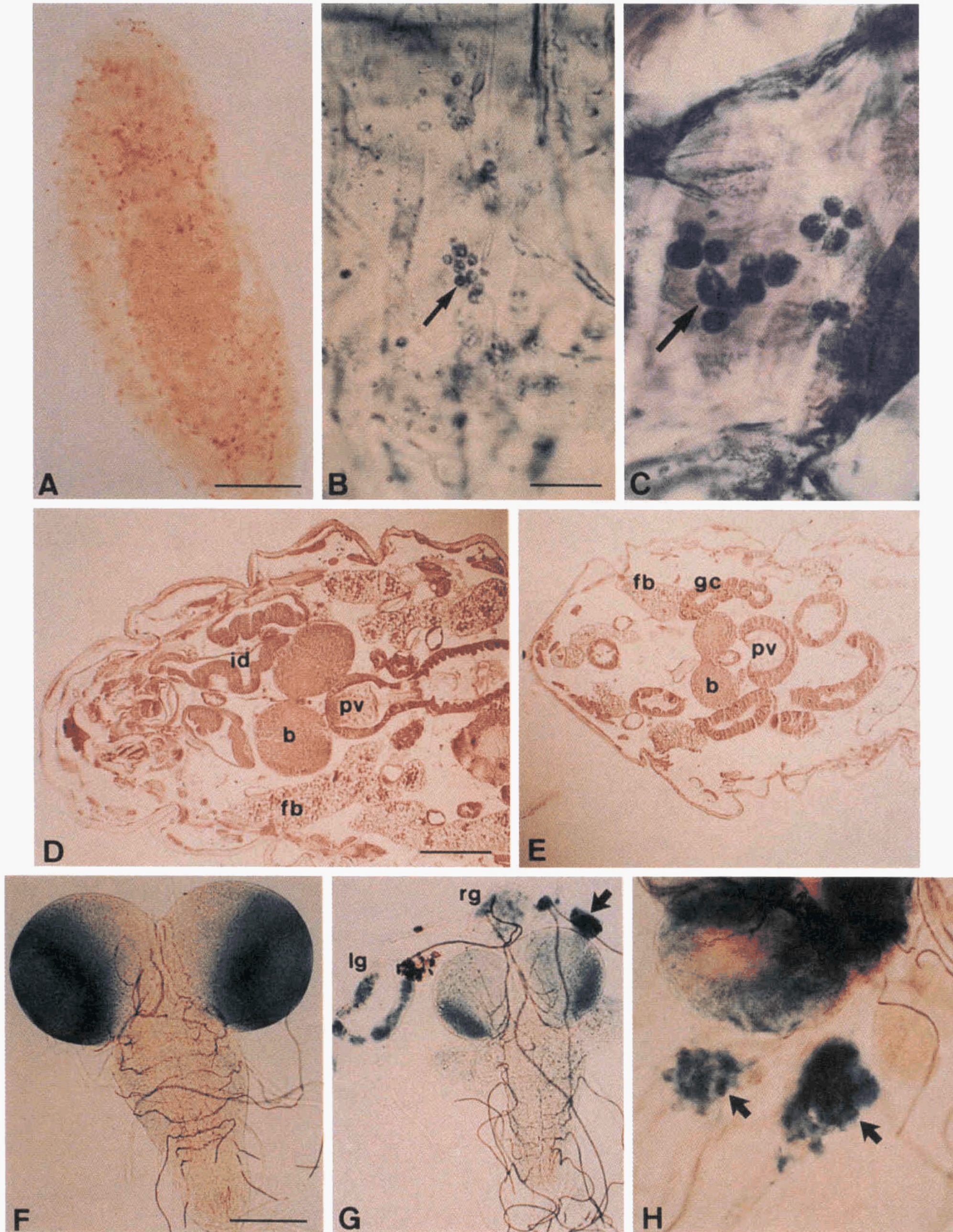

FIGURE 6.—The *domino* phenotype. (A) The distribution of hemocytes in *domino* embryos is comparable to wild type. Hemocytes are stained with an antibody against Croquemort that is expressed from stage 13 of embryogenesis. In this experiment, a double-staining with anti-croquemort and anti-β-galactosidase antibodies allowed the recognition of *domino* homozygous embryos by the absence of *elav-lacZ* staining. The *domino P-lacZ* is not expressed at this stage. Bar, 100 μm. (B and C) Whole mounts of integument inner wall from wild-type (B) and *domino* (C) first instar larvae. Groups of hemocytes (arrows) are visible on muscle and epithelial cells. Histochemistry: toluidin and eosin. Bar, 20 μm for B and C. (D and E) longitudinal sections of the anterior region of a wild-type (D) and a *domino* (E) third instar larva, showing the absence of normal imaginal discs in the mutant. Histological preparation: Hansen's haematoxylin and erythrosin. b, brain; fb, fat body; gc, gastric caeca; id, imaginal discs; pv, proventriculus. Bar, 200 μm for D and E. (F–H) Brain preparations from a heterozygous esg^{P3} (F) and esg^{P3}, *domino/domino* (G and H) third instar larvae, stained for *lacZ* activity. The *esg* expression is observed in rings of cells surrounding the optic lobes and corresponds to the neuroblast region. The *domino* mutant brain is smaller than wild type, which is probably due to reduction of the neuroblast region. Small and abnormal imaginal discs can be seen attached to the brain (arrows) and contain cells that are considerably enlarged (H). The staining in the ring gland (rg) and in the lymph glands (lg) is due to the *domino P-lacZ*. Bar, 100 μm for F and G, H is 2.5 times larger. In A, anterior is to the top; in D and E anterior is to the left.

the brain and the oenocytes, all tissues express *lacZ* more frequently in the hemocyte-staining pool than in the general staining pool (compare Figure 1B and Table 2). This enrichment is especially striking for the lymph glands (3–4% in the general pool, 50% in the hemocyte-staining pool).

TABLE 5

lacZ expression in *domino* mutants

	Larvae	
	Heterozygous	Homozygous
Discs	(+)	Absent
Ring gland	(+)	(+) reduced
Salivary glands	(+)	Reduced
Gut	(+)	+
Lymph gland	(+)?	+
Brain	NS	Reduced
Hemocytes	NS	Absent

lacZ expression in *domino* mutants. Staining intensities are given as follows: +, intermediate staining; (+), weak staining; NS, nonstaining. In homozygous larvae, the organs that are either absent or reduced are indicated.

Three of the lines analyzed in this screen appear of particular relevance in the context of Drosophila host defense:

1. Line *l(3)03349* shows *lacZ* expression in hemocytes at all stages of development. In third instar larvae, it exhibits a strong staining in lamellocytes, which is the predominant staining at this stage. *lacZ* is also expressed in embryonic and in adult hemocytes in this line (not shown).
2. Line *l(3)03550* stains strongly in most plasmatocytes, in lamellocytes and in the lymph glands. A high percentage of homozygous third instar larvae present a melanotic tumor phenotype that is possibly linked to a disregulation of blood cell function as these tumors are generally free floating in the hemocoel and are not associated with a specific tissue. They could correspond to class II tumors as defined by WATSON *et al.* (1991), *i.e.*, tumors resulting from abnormal blood cells that attack normal tissues. The analysis of these two lines is underway.
3. Line *l(2)81/8* carries a mutation named *domino* that results in a total absence of circulating hemocytes in homozygous third instar larvae and is however perfectly viable until pupariation. The *domino* mutation will be a powerful tool to investigate the role of blood cells in the different facets of the immune response, as well as in the formation of melanotic tumors. The *domino* mutant is distinct from the only other blood cell deficient mutant described so far, where the EMS-generated mutation was mapped to the third chromosome. This mutant, referred to as *l(3)hem* for *hematopoiesis missing* (GATEFF 1994), is devoid of lymph glands. It also exhibits an abnormal brain with giant neuroblasts that do not divide, and abnormal imaginal discs. E. GATEFF proposed that the *l(3)hem* gene product is involved in cell type-specific inhibition of cell division. In *domino* mutant larvae, all diploid tissues are affected to various extents: imaginal structures, neuroblasts, germline cells, and blood cells. This phenotype, together with late lethality, is typical for mutations in genes involved in cell proliferation. It has been hypothesized that most, if not all, divisions needed to form a larva (at least the 13 first rounds of embryonic mitosis that occur in the almost complete absence of zygotic transcription) are accomplished using maternal products packaged into the egg (see GATTI and GOLDBERG 1991, for a review). In the *domino* mutant, this maternal supply could explain the normal presence of embryonic macrophages. Anomalies develop later in the various tissues that maintain mitotic activities in larvae. Cell death that occurs in the hematopoietic organ thus results in the absence of hemocytes in the mutant larvae. We are currently characterizing the gene corresponding to the *P*-element insertion that we have localized at position 57E1-2.

We have used the technique of enhancer detector to find new genes that are possibly involved in immune functions of Drosophila larvae. A similar approach was recently described by RODRIGUEZ *et al.* (1996), but the lines they identified correspond to different insertions as the screening methods were different. RODRIGUEZ and coworkers looked for *lacZ* expression in embryonic lymph gland and/or hemocytes, and for melanotic tumor phenotypes or upregulated β-galactosidase activity in larvae. Among the lines they screened, one fly line was identified in which the β-galactosidase activity was increased about twofold after bacterial challenge. We did not find any immune-induced reporter gene expression in the 1341 lines that we analyzed. These results are somewhat surprising as at least 10–20 genes are known to be either turned on, or upregulated by septic injury and we suspect that there could be a bias in the enhancer trap approach for detection of immune-induced *lacZ* expression.

RODRIGUEZ and coworkers isolated six novel transposon-containing genes that are potentially involved in immunity or tumor formation, plus a previously identified gene encoding collagen IV. These two independent enhancer trap studies in the field of Drosophila immunity provide new molecular tools to investigate the mechanisms that underly immune reactions and self/non-self recognition in innate immunity.

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