Genes Expressed in the Ring Gland, the Major Endocrine Organ
of *Drosophila melanogaster*

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ABSTRACT

We have used an enhancer-trap approach to begin characterizing the function of the Drosophila endocrine system during larval development. Five hundred and ten different lethal *PZ* element insertions were screened to identify those in which a reporter gene within the *P* element showed strong expression in part or all of the ring gland, the major site of production and release of developmental hormones, and which had a mutant phenotype consistent with an endocrine defect. Nine strong candidate genes were identified in this screen, and eight of these are expressed in the lateral cells of the ring gland that produce ecdysteroid molting hormone (EC). We have confirmed that the genes detected by these enhancer traps are expressed in patterns similar to those detected by the reporter gene. Two of the genes encode proteins, protein kinase A and calmodulin, that have previously been implicated in the signaling pathway leading to EC synthesis and release in other insects. A third gene product, the translational elongation factor EF-1α F_1, could play a role in the translational regulation of EC production. The screen also identified the genes *couch potato* and *tramtrack*, previously known from their roles in peripheral nervous system development, as being expressed in the ring gland. One enhancer trap revealed expression of the gene encoding the C subunit of vacuolar ATPase (V-ATPase) in the medial cells of the ring gland, which produce the juvenile hormone that controls progression through developmental stages. This could reveal a function of V-ATPase in the response of this part of the ring gland to adenotropic neuropeptides. However, the gene identified by this enhancer trap is ubiquitously expressed, suggesting that the enhancer trap is detecting only a subset of its control elements. The results show that the enhancer trap approach can be a productive way of exploring tissue-specific genetic functions in Drosophila.

I N insects, as in all animals, many aspects of development are under hormonal control. The most important insect hormones are the ecdysteroid molting hormone (EC), which is secreted from the prothoracic glands and the sesquiterpenoid juvenile hormone (JH), which is secreted from the corpus allatum (CA). The generally accepted view is that ecdysteroid peaks determine the time of molting from one instar to the next, whereas JH levels determine whether the animal molts to a larval, pupal, or adult form.

The details of the neuroendocrine control of insect development have been best characterized in lepidopterans. Many, but not all, of the biosynthetic steps and intermediates leading from dietary cholesterol to the biologically active EC 20-hydroxyecdysone (20-HE) have been identified (Grieneisen 1994). EC receptors and numerous EC-responsive genes have been identified, and progress is being made in understanding the molecular nature of the EC response (Cherbas and Cherbas 1996). One EC peak early in the last larval instar (the "commit-

ment peak") apparently causes the epidermis to become committed to producing either larval or pupal cuticle at the next molt, whereas a later, sharper EC peak (the "prepupal" or "molting peak") is responsible for initiating the molt itself (Riddiford 1978). Five JHs have been identified in lepidopterans, and shown to be sesquiterpenoids that are synthesized from acetate and/or propionate in the CA (Gilbert *et al.* 1996). The main form in Drosophila is methyl 6,7;10,11-bisepoxy-3,7,11-trimethyl-(2E)-dodecenoate (JHB3; Richard *et al.* 1989).

The levels of EC and JH are regulated by adenotropic neuropeptides that are produced in the developing brain and delivered to the endocrine glands via the axons of neurosecretory cells (Gilbert *et al.* 1996). Some of the neuropeptides (large and small prothoracicotropic hormone or PTTH) stimulate EC production by the prothoracic glands, whereas others either stimulate [allatotropic hormone or allatotropin (ATH)] or inhibit [allatostatic hormone or allatostatin (ASH)] JH production by the CA. Immunohistochemical studies in both lepidopterans (Mizoguchi *et al.* 1987) and Drosophila (Zitnan *et al.* 1993) show that each of these peptides is produced by a small number of neurosecretory cells located in defined positions of the developing brain.

The signaling pathways leading from the adenotropic neuropeptides to the synthesis and release of EC and JH

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have been investigated most extensively in the tobacco hornworm *Manduca sexta*. For the commitment peak of EC occurring during mid-fifth larval instar, PTTH appears to act via a Ca^{2+}/calmodulin-dependent cAMP pathway leading to the phosphorylation of a specific set of proteins including ribosomal protein S6 and β-tubulin (Smith 1995; Gilbert *et al.* 1996). The mechanisms involved in the stimulation of molting and metamorphosis by the later, larger peak of EC are not yet clear. In studies of the control of JH production by the CA, Reagan *et al.* (1992) showed that ATH induces phosphoinositide hydrolysis and that inhibition of Ca^{2+}-ATPase, protein kinases A and C, and ATP-dependent Ca^{2+} sequestration inhibited production of the hormone. These results suggest that the inositol 1,4,5-triphosphate pathway may be involved in the response to ATH and possibly other neuropeptides.

The difficulty of genetic analysis in lepidopterans has restricted the possibilities for functional analyses of the neuroendocrine pathways controlling the development of these insects. However, similar neuroendocrine mechanisms appear to operate in dipterans including Drosophila, an insect for which excellent molecular and genetic techniques have been developed. In higher dipterans, the larval prothoracic glands, CA, and corpus cardiacum are fused into a single compound structure, the ring gland (King *et al.* 1966) which has been shown experimentally to produce both EC (Roberts *et al.* 1984) and JH (Richard *et al.* 1989). Based mainly on morphological homologies between the ring gland and the endocrine glands of larger insects, it is thought that EC is produced by the large lateral cells (LC) homologous to the prothoracic glands of other insects and JH is produced by the smaller medial cells (MC) homologous to the CA. Ventral ganglion/brain complexes of wandering third-instar Drosophila larvae show PTTH activity in vitro (Henrich *et al.* 1987) and two forms of PTTH separable by molecular weight have been isolated from late third-instar brains (Pak *et al.* 1992). A 66-kD glycoprotein with PTTH activity on ring glands *in vitro* has been purified from Drosophila larvae but it has no significant homology to known PTTH peptides (Kim *et al.* 1997). Furthermore, Drosophila late-larval brain extract stimulates EC production from *M. sexta* prothoracic glands, and partially purified large and small *M. sexta* PTTH stimulate EC production from Drosophila ring glands (Henrich 1995). Antibodies made against *B. mori* large and small PTTH stain Drosophila neurosecretory cells in a spatiotemporal manner similar to that seen in lepidopterans (Zitnan *et al.* 1993). Antibodies to *M. sexta* ASH and ATH also cross-react in specific neurosecretory cells of larval fly brains but the correlation to lepidopteran patterns is less clear. An ASH activity also has been described from the Drosophila brain (Richard *et al.* 1989; Richard *et al.* 1990). Taken together, these results strongly suggest a molecular conser-

vation of basic neuroendocrine processes between moths and flies, with some order-specific modifications.

In the present study, we have used the "enhancer-trap" technique (O'Kane and Gehring 1987; Grossniklaus *et al.* 1989; Wilson *et al.* 1989; Bellen *et al.* 1989) to identify Drosophila genes that are strongly expressed in all or part of the ring gland during development. With this method, a reporter gene encoding β-galactosidase under the control of a weak promoter, namely the P transposase promoter, is carried to various genomic sites by a transposable element. When the reporter gene shows expression in the ring gland, it is probably inserted into or near a gene with a similar expression pattern (Wilson *et al.* 1989; Bier *et al.* 1989). We have restricted our attention to lines in which the insertion is lethal so that analysis of the terminal phenotype might provide clues to the disrupted gene's function. Similar techniques have been used to detect genes expressed in other tissues and/or during specific developmental stages (*e.g.*, Mlodzik and Hiromi 1992), but the ring gland has not been studied in previous screens. By focusing on the ring gland and screening for phenotypes suggestive of neuroendocrine defects, we hope to identify genes involved in the signaling or biosynthetic pathways downstream of the adenotropic hormones as well as those required for the development of the ring gland.

MATERIALS AND METHODS

Drosophila stocks: Lethal *PZ* insertional mutations (Mlodzik and Hiromi 1992) were generated in the laboratory of Dr. Allan Spradling (Karpen and Spradling 1992) and provided to us by Dr. John Tower at the University of Southern California (Los Angeles). Fly cultures were maintained on standard cornmeal-molasses-agar medium at 25° unless otherwise stated. Mutant chromosomes were balanced over *CyO* or *In(2LR)Gla, Gla Bc Elp* (second-chromosome lines) or *In(3LR) TM3 y^+ ri p^p sep bx^{34e} e^s* (third-chromosome lines).

5-bromo-4-chloro-3-indoxyl-β-d-galactopyranoside (X-Gal) staining: Tissue was dissected in Ringer solution or phosphate-buffered saline (PBS), fixed in 1% glutaraldehyde for 15–30 min at room temperature, and incubated in 2% X-Gal (Gold Biotechnology, St. Louis, MO) dissolved in *N,N'*-dimethylformamide diluted 1:10 in staining buffer (10 mm sodium phosphate, 3.1 mm ferropotassium cyanide, 3.1 mm ferripotassium cyanide, 150 mm sodium chloride, 1 mm magnesium chloride) for up to 12 hr at room temperature.

Antibody staining: Tissue was dissected in Ringer solution or PBS, fixed in 4% paraformaldehyde for 20 min at room temperature and incubated in a monoclonal antibody to β-galactosidase (Promega, Madison, WI) diluted to 1 μg/ml in PBS plus 0.1% Triton X-100 (Fisher Scientific, Pittsburgh, PA) for 1 hr at room temperature. Tissues were incubated in a biotinylated anti-mouse IgG secondary antibody and immunoreactivity was visualized using Vectastain *Elite* and Vector VIP kits (Vector Laboratories, Burlingame, CA).

Plasmid rescue: Adult flies were ground in lysis buffer (0.32 mM sucrose, 10 mm TRIS pH 8.0, 5 mm $MgCl_2$, 1% Triton-X in TBS), filtered through Nytex cloth, and centrifuged at 1100 g at 4° for 12 min. The pellet was resuspended in 75 mm NaCl, 24 mm EDTA and digested with 4 μg/fly equivalent

Proteinase K (BRL, Hercules, CA) plus 1% Triton-X at 37° for 2–18 hr. Following phenol/chloroform extraction, the DNA was precipitated in KCl/ethanol. The pellet was resuspended in TE and 3–4 μg of DNA was digested with *Xba*I or *Xba*I and *Not*I (Promega), phenol/chloroform extracted, NaOAc/ethanol precipitated, and resuspended in dH₂O. Ligation of 1–2 μg of digested DNA was performed in 200 μl volume using 1–3 units T4 DNA ligase (Promega) at 14° for 4–5 hr. Following phenol/chloroform extraction and NaOAc/ethanol precipitation, the ligated DNA was transfected into DH5α *Max Efficiency* competent cells (BRL) which were then plated on 50 μg/ml kanamycin NZCYM plates for plasmid rescue (Pirrotta 1986). DNA isolated from individual colonies was checked for *PZ* elements by restriction mapping of miniprep DNA with *Not*I, *Xba*I, and *Not*I/*Xba*I (Sambrook *et al.* 1989; Mlodzik and Hiromi 1992).

Polymerase chain reaction (PCR): All PCR reactions were performed in a thermocycler (MJ Research, Inc., Watertown, MA) according to standard protocols (Innis *et al.* 1990) using *Taq* DNA polymerase (Promega), dNTPs (Epicentre Technologies Corp., Madison, WI), and oligonucleotide primers (Operon Technologies, Inc., Alameda, CA). When the gene identity was known from published data, primers were based on sequences at the end of the *PZ* insert and on published sequence from the affected gene. The latter included the genomic region adjacent to the *PZ* insertion in *l(2)1275* (available from the Berkeley Drosophila Genome Project [Berkeley, CA, 1998 at http://fruitfly.berkeley.edu/], and for the EF-1α F₁ gene [Hovemann *et al.* 1988; Flybase 1998 at http://flybase.bio.indiana.edu.82/]). DNA was amplified, subcloned into the TA vector (Invitrogen, San Diego), and sequenced.

Library construction and screening: For *l(2)6072*, DNA adjacent to the *PZ* insertion was cloned from a genomic DNA library constructed from adult flies. Following partial digestion with *Sau3A* and A/G nucleotide fill-in, DNA was ligated into the Lambda Fix II vector and packaged with the Gigapack III Gold packaging extract (Stratagene, La Jolla, CA). From approximately 1.5×10^5 pfu of unamplified library plated with NM538 bacteria on NZCYM plates, a single clone was plaque-purified (Sambrook *et al.* 1989) using a random-primed radiolabeled probe (Prime-It; Stratagene) complementary to a 400 bp PCR product containing genomic DNA adjacent to the *PZ* insertion in *l(2)6072* (see above).

The transcription units corresponding to *l(2)4524* and *l(2)6072* were cloned from cDNA libraries. Two third-instar central nervous system (CNS)/ring-gland cDNA libraries, one constructed in the EMBL4 vector and one in the Lambda ZAP vector (Stratagene), were screened using standard protocols (Sambrook *et al.* 1989). In each case, plaque purification began with an initial hybridization to 8×10^6 pfu plated with NM538 bacteria on NZCYM plates. Random-primed radiolabeled probes (Prime-It; Stratagene) were made using 3.0-kb and 1.4-kb fragments adjacent to the *PZ* insertions of *l(2)4524* and *l(2)6072*, respectively.

Subcloning and sequencing: For sequencing, four *Hin*dIII fragments representing the 3.0 kb adjacent to the *PZ* insertion in *l(2)4524* were subcloned into the Bluescript SK+ plasmid (Stratagene), and the PCR products generated for *l(2)1275* were subcloned into the TA vector (Invitrogen). All other sequencing of genomic DNA was performed directly from the rescued *PZ* plasmids. Sequencing of cDNAs was carried out either directly from purified phage DNA (Sambrook *et al.* 1989) or, in the case of the Lambda ZAP clones, from excised phagemid DNA (Stratagene). All sequencing was carried out as described for the dsDNA Cycle Sequencing System (BRL) using radiolabeled dCTP (New England Nuclear, Boston).

Lethal phase analysis: The lethal phase(s) for 15 second-chromosome *PZ* insertion lines balanced over *In(2LR)Gla, Gla*

Bc Elp were determined as follows: Larvae from 1.0–1.5 hr egg collections were allowed to develop at 25° until mid-third instar and transferred to 18° for 16 hr to enhance the Black Cell (*Bc*) phenotype. Larvae were scored as heterozygous or *PZ*-homozygous by the presence or absence of black cells, respectively. Since *Bc/Bc* homozygotes die as first instars, a 2:1 ratio of third-instar heterozygotes to homozygotes was taken as evidence that neither significant lethality nor developmental delay had occurred in insertion homozygotes prior to third instar. Homozygotes were placed on fresh food and allowed to develop at 25°. Larvae were checked twice daily and the number of live and dead animals and their developmental stage scored. Examining the animals for up to 14 days allowed the detection of abnormal developmental timing as well as determination of lethal phase.

Whole-mount RNA *in situ* hybridization: CNS/ring gland complexes were dissected into PBS, fixed in 4% paraformaldehyde, PBS, 0.1% Tween 20 for 20–30 min, and rinsed in PBS, 0.1% Tween three times for 20 min each. Tissue was pretreated with Proteinase K (Promega) and hybridized as described by Tautz and Pfeifle (1989) except that hybridization was carried out for 1 day at 65° and washes lasted for 2 days at 65°. RNA probes were generated using the T3 and T7 promoters and the DIG Labeling Mixture (Boehringer Mannheim, Indianapolis), and were boiled for 30 min prior to use. Hybridization was detected with a monoclonal antibody against digoxygenin coupled to alkaline phosphatase (Boehringer Mannheim).

RESULTS

Enhancer-trap screen: We screened 510 lethal *PZ* enhancer-trap lines, in which the recessive lethality had previously been shown to be caused by the enhancer-trap insertion (Mlodzik and Hiromi 1992; A. Spradling, personal communication). We selected those showing strong β-galactosidase reporter gene activity as detected by X-Gal staining in the ring gland of wandering third-instar larvae. We have not excluded lines showing expression outside the ring gland, because in other insects there is evidence for production of EC in tissues other than the prothoracic glands (Rees 1985). Since the β-galactosidase protein is fairly stable in vivo, assaying wandering third-instar larvae allowed us to identify enhancer-trap insertions into or near to genes expressed in the ring gland at other times during the third instar including presumably the times of the commitment and prepupal peaks of EC.

X-Gal staining in the ring gland was detected in 76/510 = 15% of lines examined (31/270 = 11% of second-chromosome and 45/240 = 19% of third-chromosome lines). In 25 of the 76 lines, the ring gland was the predominant or most darkly staining tissue (Table 1), but in only three lines was X-Gal staining completely restricted to the ring gland at this stage. Significant staining occurred in the CNS in 70 lines and in one or more pairs of imaginal discs in 59 lines.

Expression profiles: Based on intensity and specificity of ring-gland staining, 18 second-chromosome and 13 third-chromosome lines were selected for a more detailed analysis (Table 2). The *lacZ* expression patterns in the ring gland, CNS, imaginal discs, lymph gland, fat

TABLE 1

Tissue staining patterns in wandering third-instar larvae of lines expressing lacZ in the ring gland

Tissues stained	Enhancer-trap location		Total No. of lines
	Second chromosome	Third chromosome	
Ring gland and:			
No other tissue	1 (2535)	2 (**2640**, **4679**)[a]	3
Nonpunctate CNS[b]	2 (**1209**, **10280**)	2 (5125, 5301)	4
Nonpunctate CNS and imaginal discs	15 (**0248**,[c] 0455, 1550, 2439, 2459, 2532, **2970**, **3041**, 3055, **3602**, **3909**, 4733, **5029**, **6072**, 10206)	16 (2017, 2414, 2515, 3540, 3544, **4136**, 4674, 4836, 5559, **6015**, 6286, **6487**, 6535, 7084, 7238, 8310)	31
Punctate CNS and imaginal discs	1 (8253)	7 (**1824**, 2331, 3527, 3603, 3702, 5822, 6524)	8
Nonpunctate CNS and other	8 (1296, 2278, 3907, **4012**, 4524, 5836, 5841, 8717)	14 (1619, 4712, 4720, **5134**, 5295, 5311, 5339, 5471, 5472, 5473, 5712, 7128, 8034, **9402**)	22
Punctate CNS and other	3 (1275, 6353, **7447**)	2 (**2299**, **8247**)	5
Other	1 (**1857**)	2 (**3520**, **6345**)	3
Total	31	45	76

[a] Bold, ring gland was the darkest or predominant tissue stained.
[b] CNS, central nervous system (optic lobes and ventral ganglion).
[c] Underline, eye discs were the only imaginal discs to stain.

TABLE 2

Staining of different parts of the ring gland in enhancer-trap lines

Ring-gland staining[a]		Second chromosome[b]	Third chromosome[b]
LC, MC, CCC	All stages	4524	5822
	Some stages	7447 (2, 3)	
LC, MC	All stages	2970, 3041, 3909, 2278	1824, 3603
	Some stages	1550 (1, 2, 3 but not 2/3)	4136 (3), 2299 (2, 3)
LC	All stages	1857, 3602, 8253, 1275, 3907, 4012, 5029, 6353, 10280	4836, 6345, 3540, 3544, 5311, 9402, 3520
	Some stages	0248 (2, 3, see text), 2438 (3)	2331 (2, 3)
MC	Some stages	6072 (2, 3)	

[a] LC, lateral secretory cells, homologous to prothoracic glands; MC, medial secretory cells, homologous to corpus allatum; CCC, corpus cardiacum cells.

[b] Parentheses, larval instars showing positive staining; 2/3, second to third instar molt.

body, and salivary glands were determined enzymatically by incubating with X-Gal and/or immunohistochemically by staining with a monoclonal antibody to β-galactosidase. Expression was assayed in first-instar, second-instar, second- to third-instar molting, and early, middle, and late third-instar larvae.

In 23 lines, β-galactosidase activity was detected in the ring gland at all stages analyzed (Table 2). Among these lines, staining was observed throughout the ring gland, *i.e.*, in the LC, MC, and the corpus cardiacum-homologous cells (CCC) located at the two horns of the ring gland, in two cases, was restricted to the LC and MC in seven cases and to the LC in 14 cases. There was significant staining in one or more pairs of imaginal discs in all 23 lines. In line *l(2)1857*, the ring gland stained darkly at all larval stages (Figure 1) while the only other significant staining was in the eye discs of late third-instar larvae. In embryos of this line, X-Gal staining marked the apparent ring-gland precursor cells as early as 5 hr after egg laying (AEL; Figure 1B).

In eight lines, β-galactosidase activity was detected in the ring gland of only some of the larval stages analyzed
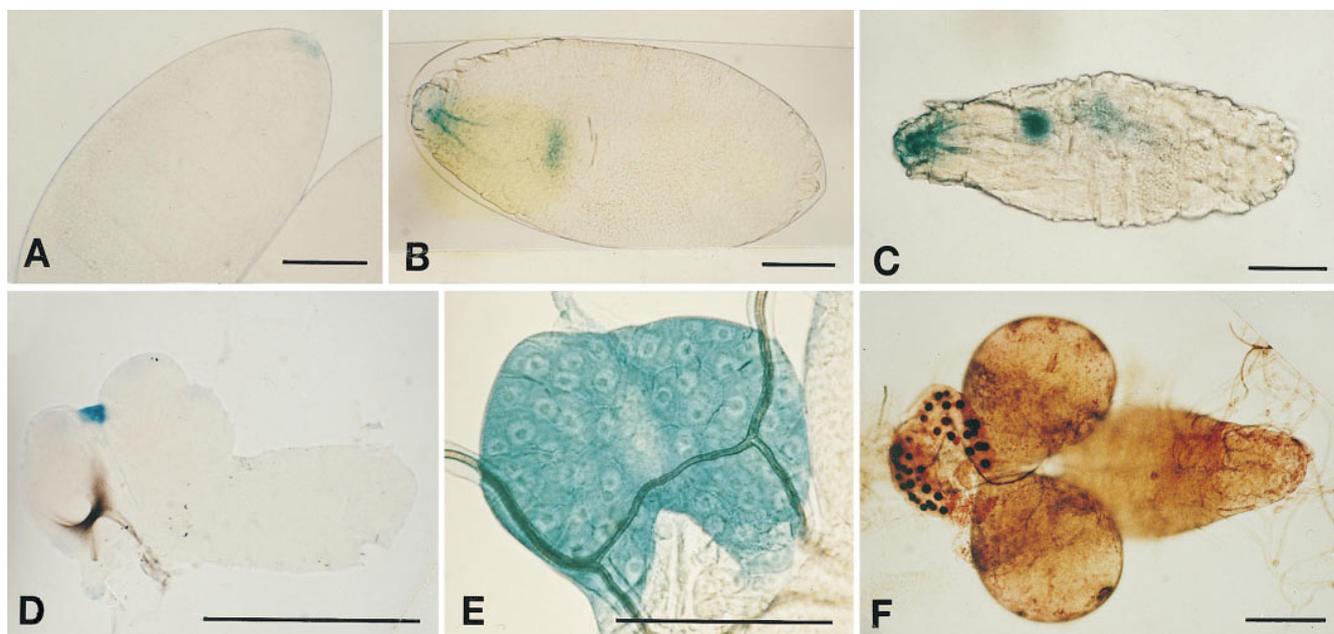

Figure 1.—Developmental profile of *l(2)1857* staining. (A–E) X-Gal staining; (F) Immunohistochemical staining with monoclonal antibody (mAb) to beta-galactosidase. (A) 1–2-hr AEL; anterior pole stained. (B) 5-hr AEL; apparent ring gland precursor cells stained. (C) Newly hatched homozygous first-instar larva; ring gland darkly stained. (D) CNS/ring gland complex from heterozygous late first-instar larva; ring gland darkly stained. (E) Third-instar ring gland; LC stain darkly but reaction product has apparently diffused into MC as well. (F) Anti-β-galactosidase staining; only nuclei of LC stain darkly. Bars, 100 μm.
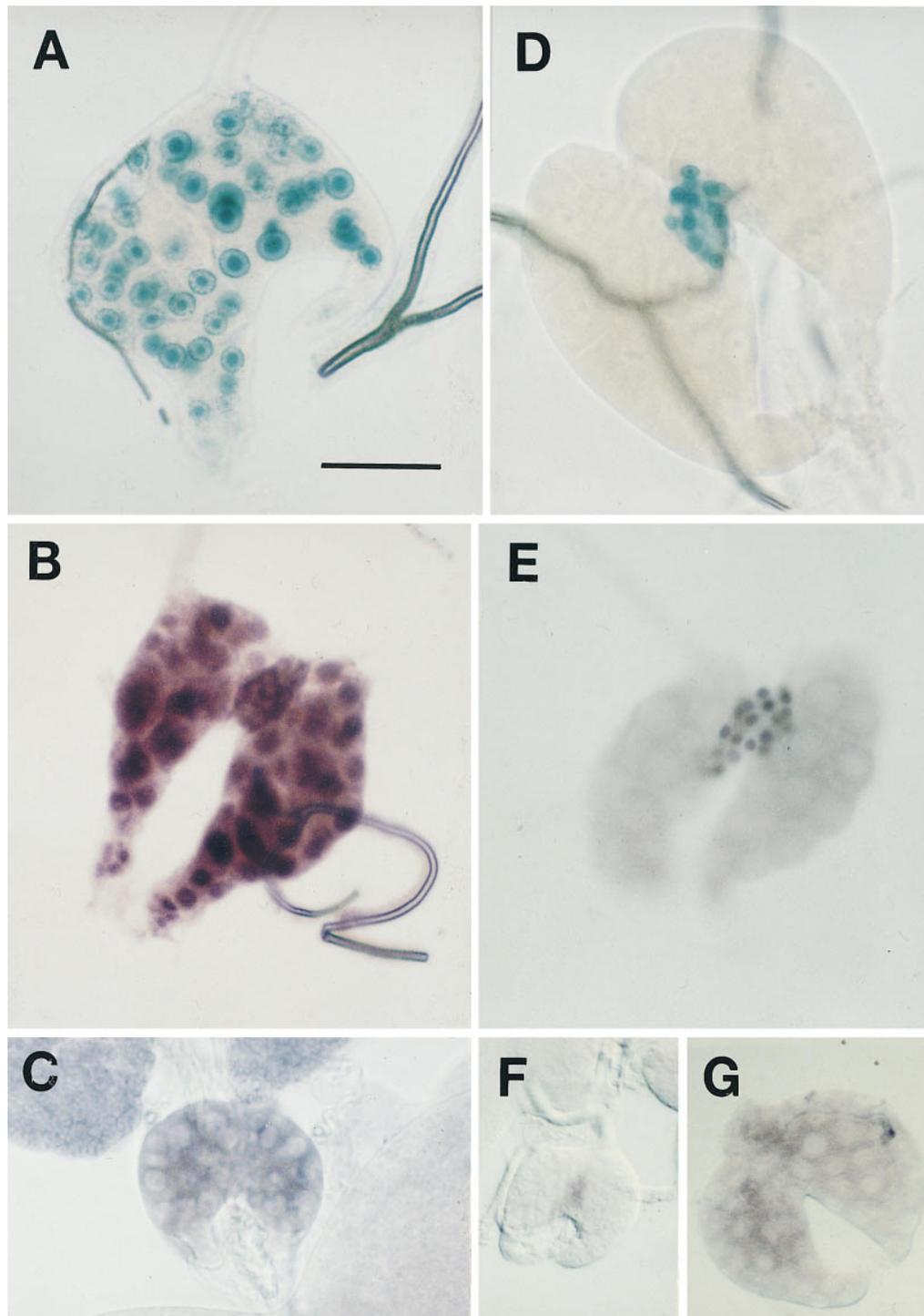
Figure 2.—(A–C) *l(2)4524* third-instar ring glands. (A) Only the LC stain with X-Gal, while both the LC and the MC stain with a mAb to β-galactosidase (B) and in RNA *in situ* hybridization using a *lacZ* probe (C). (D–G) *l(2)6072* third-instar ring glands. Only the MC stain with X-Gal (D), a mAb to β-galactosidase (E), and in RNA *in situ* hybridization using a *lacZ* probe (F). However, RNA *in situ* hybridization using a V-ATPase C subunit probe shows that a nearby endogenous gene is expressed throughout the entire ring gland (G) as well as in other tissues. All ring glands were of similar size when dissected from larvae. Bar, 50 μm.

(Table 2). In *l(2)0248*, staining was detected in the ring glands of second-instar larvae prior to but not during molting, and again in mid- and late third-instar larvae, the times that immediately precede EC peaks. In the remainder of the lines, staining was observed in late larval stages but not earlier ones. All ring gland cells stained in one line, and staining was restricted to the LC and MC in four lines and to the LC in three lines (Figure 2A). In two lines, *l(2)2535* and *l(2)6072*, *lacZ*

expression within the ring gland was clearly restricted to the JH-producing MC (Figure 2, D and E). All but one line, *l(2)7447*, also showed staining in imaginal discs.

Isolation and characterization of genomic DNA adjacent to the *PZ* insertion: The genomic DNA adjacent to the *PZ* insertion was isolated from five second-chromosome and five third-chromosome lines, *l(2)3909*, *l(2)4012*, *l(2)4524*, *l(2)6353*, *l(2)7447*, *l(3)3540*, *l(3)3544*, *l(3)5822*, *l(3)6015*, and *l(3)6286*, by plasmid rescue, from line

TABLE 3

***PZ* enhancer-trap insertions into or near to Drosophila genes or elements**

Line	Gene or element	Reference	Insertion site	Orientation[a]	Expression stage; cells[b]	Lethal phase[c]
l(2)3909	Calmodulin	Doyle *et al.* 1990	60 bp 5′ to transcription start site	+	All; LC+MC	Ph+A
l(2)6353	DC0 (PKA)	Kalderon and Rubin 1988	After position 71 of 5′ untranslated region	+	All; LC	2nd/3rd
l(2)1275	EF-1α F$_1$	Hovemann *et al.* 1988	30 bp 5′ to transcription start site	−	All; LC	3rd
l(2)4524	66.5-kD protein	Wilson *et al.* 1994	445 bp 3′ to poly A site	+	All; All	Ph+A
l(3)3540	*tramtrack*	Giesen *et al.* 1997	First intron	+	All; LC	E[n]
l(3)6015	*couch potato*	Bellen *et al.* 1992	37 bp 5′ to transcription start site	−	All; All	E[d]
l(2)2278	*expanded*	Boedigheimer and Laughon 1993	ND[e]	ND	All; LC+MC	Pu
l(2)4012	1360 element	Kholodilov *et al.* 1987	Within 1360 element	NA[f]	All; LC	A
l(2)6072	V-ATPase C subunit	Van Hille *et al.* 1993	5′ to coding sequence	−	2nd–3rd; MC	3rd

[a] Orientation of *PZ* element relative to endogenous gene; +, *lacZ* reporter and endogenous genes transcribed in opposite directions.

[b] Stage at which ring gland shows expression; LC, lateral secretory cells; MC, medial secretory cells.

[c] E, embryonic; 2nd, 3rd, larval instars; Pu, pupa; Ph, pharate adult; A, adult.

[d] From published descriptions of amorphic allele homozygotes.

[e] Not determined.

[f] Not applicable.

l(2)1275 by PCR utilizing sequence data available from the Berkeley Drosophila Genome Project and from line *l(2)6072* by screening a genomic library made from that line. *l(2)6072* shows expression in the MC, whereas the remaining lines show expression in the LC or both LC and MC. That the genomic DNA was in fact immediately adjacent to the *PZ* insertion was confirmed by sequencing across the insertion/genomic DNA boundary using an internal enhancer-trap primer. We also sequenced the initial 200–700 bp of genomic DNA from each isolate. These sequences have been deposited in GenBank. BLAST searches (Altschul *et al.* 1990) indicated that for six of the sixteen lines analyzed, the *PZ* insertion is in or near a previously cloned Drosophila gene or element (Table 3). The insertion is located within 60 bases 5′ of the transcription start site in three of these, 71 bases into the 5′ untranslated region in one, and into the intron in another.

l(2)3909: The extended third-instar phenotype of *l(2)3909*, in which the *PZ* insertion is 60 bp 5′ to the transcriptional start site of the calmodulin (*Cam*) gene, is similar to that reported for EMS-induced *Cam* alleles (K. Beckingham, personal communication). Homozygous *Cam* null animals die as first instar larvae (Heiman *et al.* 1996) but in the heteroallelic condition with either of two EMS-induced alleles they produce animals that survive to pupae. The extended third larval instar seen in these heteroallelic combinations (K. Beckingham, personal communication) is suggestive of an endocrine

defect. The *l(2)3909 lacZ* and wild-type *Cam* expression patterns are very similar or identical for all stages of development from embryo to late third instar (Kovalick and Beckingham 1992; K. Beckingham, personal communication). This pattern is dynamic during the third instar. In early third-instar larvae, expression is limited to and very robust in the ring gland but by the prepupal stage it has expanded to include most tissues (Figure 3, E–G). We were unable to definitively determine by RNA *in situ* hybridization whether *Cam* expression within the ring gland is limited to the LC or whether it also includes the MC, but immunohistochemical localization of β-galactosidase indicated that the reporter gene is expressed in both the LC and the MC.

l(2)6353: The enhancer trap of *l(2)6353* is inserted into the 5′ untranslated region of the gene encoding the DC0 catalytic subunit of cAMP-dependent protein kinase (cAMP-PKA or PKA) (Lane and Kalderon 1993). Homozygotes of *l(2)6353* display slow development, molting to third instar one day later than heterozygous siblings, and most die as third-instar larvae. Many of the larvae surviving to third instar remain as such for up to eight days and initiate wandering behavior but never pupariate. Approximately 15% of the homozygotes, however, do form small pupae within 48 hr of their heterozygous siblings, and about two-thirds of these survive to eclose as miniature adults with unexpanded wings. A simple explanation for this is that PKA is involved in the signal transduction pathway leading to the
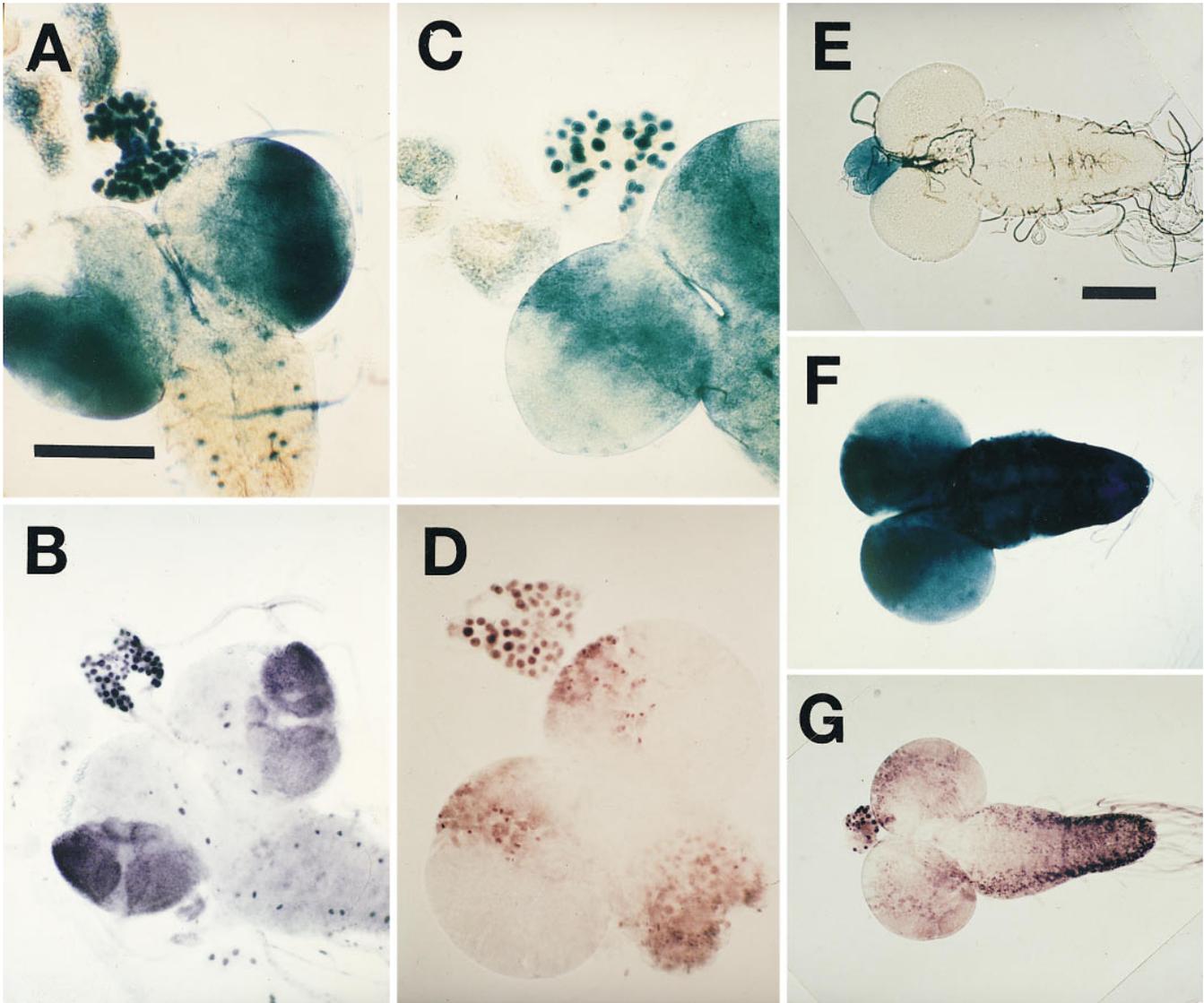
Figure 3.—A comparison of *lacZ* expression patterns as detected by X-Gal staining and by mAb staining: (A) *l(2)2278* X-Gal and (B) mAb; (C) *l(2)1275* X-Gal and (D) mAb. The dynamic pattern of expression in *l(2)3909* is shown in X-Gal staining of (E) early and (F) late third-instar CNS (ring gland removed in F). (G) mAb staining of *l(2)3909* wandering third-instar CNS/ring gland complex.

large prepupal EC peak, and that in the nonpupariating animals the signal is not sufficient to produce the peak. Since *l(2)6353* does not appear to be a null mutation for cAMP-PK, it is possible that some animals can generate sufficient signal to produce the EC required to pupariate, accounting for the escapers produced in this line.

The developmental and morphogenetic defects in *l(2)6353* are similar to those described for weak DC0 mutants in Drosophila (Lane and Kalderon 1993). It seemed likely that *l(2)6353* would be a null mutant since the enhancer trap is inserted into the 5′ transcribed but untranslated region of DC0, thereby separating the transcriptional start site from the coding region by about 14 kb that has previously been shown to contain termination and aberrant splicing signals (Horowitz and Berg 1995). Perhaps a less efficient secondary start site is utilized or a less translatable message is produced from the DC0 locus containing the *PZ* insert, resulting in a weak rather than a null mutation.

l(2)1275: A phenotype suggestive of an endocrine defect was observed in line *l(2)1275*, in which the *PZ* insertion is 30 bp 5′ to the transcriptional start site of the gene encoding the translational elongation factor EF-1α F_1 (Hovemann *et al.* 1988). Homozygotes exhibit slow growth, and molt to third instar approximately one day later than sibling heterozygotes. Ten to 15% of those surviving to third instar remain alive for up to 5 days and initiate wandering but never pupariate. There are two EF-1α genes in Drosophila but only the F_1 gene potentially disrupted here is expressed at high levels during larval development (Hovemann *et al.* 1988).

l(2)4524: Four *Hin*dIII fragments representing 3.3 kb

A l(2)4524 plasmid rescue
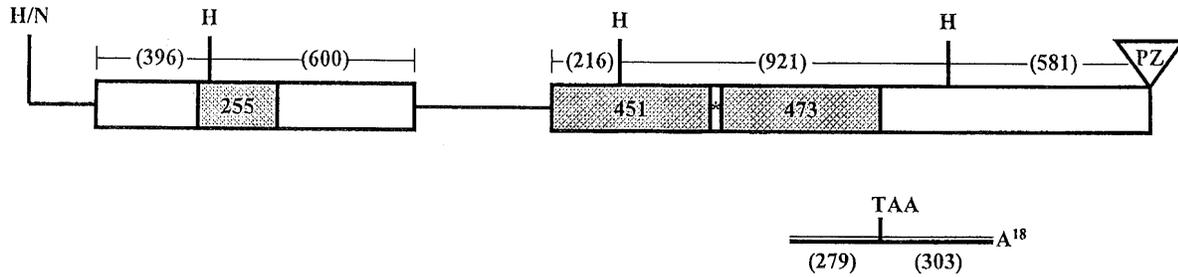
B Putative 53bp intron

GTAAGTTGATATGTGGAGTGGCTGGCGATTAAATAATGAATCGATCTATGTAG

C l(2)4524/66.5KD alignments

5' region:

```
l(2)4524   ENAREMARLVGDLRNFTSQVLKGGGQKAIERHTSRGKLLARERINLLLDKGSPGLELSAL   60
66.5KD     ANTAEMKVLVEDLKAKISKIEQAGGEKAVKLHRSRGKMLARERIDGIVDAGSPFIEFSQL   60


l(2)4524   AGHEIYGEEVVNSGGIVTGVGRVCg   85
66.5KD     AGYEMYGKEEVPSGGILTGVGIVSg   85
```

3' region:

```
l(2)4524   VTDHYAVDDEHALYLARQIvsnlnlsatnsyndqlmhssqvnfqtatpPSAVEEPRYDAR   60
66.5KD     VTDYYAHNDKHALYLARSCiaglppveehmtf----------------NPNADEPLYPAE   44


l(2)4524   ELYGIVGPNLTKsfDVREVIARIVDGSRFTEFKKLYGETLVCGFANVYGHTVgivgNNGV   120
66.5KD     EIYGIVGSNLKKtyDVREVIARIVDGSRFHEFKERYGETLVTGFATIYGQRVgilaNNGV   104


l(2)4524   LFSESALKGAHFIQLCAQRKIPLVFLQNITGFMVGRDAEANGIAKNGAKMVTAVACANVP   180
66.5KD     LFAESAMKGSHFIELCCQRKIPLLFLQNITGFMVGRDAEAGGIAKHGAKLVTAVACAKVP   164


l(2)4524   KFTVIIGGSYGAGNYGMCGRAYSPRFLYMWPNSRISVMGGTQAANVMAQITEDQRkraGK   240
66.5KD     KITVLVGGSYGAGNYGMCGRGYSPRYVFMWPNSRISVMGGEQAANVLSTVQKEKKkreGA   224


l(2)4524   EFSEEEAQKLKAPIVEMFeaeGSPYYSTARLWDDGIIDPANTRQILGlsLKAALNNAGQE   300
66.5KD     DWTDQQDLELRKPVEEKFekeGHPYFASARLWDDGVIDPKDTRKVLGlaFQSTLQKPIPE   284


l(2)4524   TKFGVFRM   308
66.5KD     TKFGVFRM   292
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Figure 4.—(A) Diagram of plasmid-rescued genomic DNA adjacent to the *l(2)4524 PZ* insertion. Regions sequenced are boxed. Regions homologous to a *C. elegans* hypothetical 66.5-kD protein are shaded. The sizes of the homologous regions are given in bases inside the boxes. H, *Hind*III site; H/N, *Hind*III or *Not*I site; *, putative intron. The position of the cDNA relative to the genomic DNA and the ORF, 3' UTR, and poly-A sizes are indicated below the diagram (double line). (B) Sequence of the putative 53-bp intron. Acceptor and donor splice sequences are double underlined. A potential TACTAAC box is in bold. (C) Macaw alignments (Schuler *et al.* 1991) of regions of homology between conceptual translations of *l(2)4524* and *C. elegans* sequences. Darkness of shading indicates the significance of the match. The putative intron sequence (B) has been removed from the 3' sequence. The region corresponding to the longest cDNA is underlined.

adjacent to the *PZ* insertion were subcloned from the genomic DNA plasmid rescued from line *l(2)4524*. The two proximal fragments of 581 bp and 921 bp and the distal fragment of 396 bp were sequenced, as were the proximal 216 bp and distal 600 bp of the fourth fragment (Figure 4A). A BLAST analysis revealed three regions of 255, 451, and 473 bp whose conceptual translations show colinear homology to a *Caenorhabditis elegans* 66.5-kD hypothetical protein (Wilson *et al.* 1994). The 255-bp region may represent a complete exon since the entire 255 bp are contained in an open reading frame (ORF) that is bounded by splice donor and acceptor sequences, and a potential TACTAAC splicing branch point (Mount *et al.* 1992) is located 35 bp upstream. The 85-amino-acid sequence encoded by this region is 55% identical to a portion of the *C. elegans* protein (Figure 4C). The other regions of homology can be resolved into a single 924-bp ORF by removal of a putative 53-bp intron that contains splice donor and acceptor sequences and a potential TACTAAC box (Figure 4B). The resulting "spliced" ORF encodes a 308-amino-acid sequence which is 61% identical to the C-terminal region of the *C. elegans* protein (Figure 4C). Approximately 8×10^6 plaques from each of two cDNA libraries, one made from the CNS of third-instar larvae and one made from the CNS and ring gland of wandering third-instar larvae, were screened using the 3 kb adjacent to the enhancer trap as a probe. Two clones were plaque purified from each library (584 and 314 bp, and 563 and 600 bp, respectively), sequenced, and shown to be from the same gene and identical to the 3′ ORF region of the rescued genomic DNA (Figure 4A). All of the cDNA clones contain a 5′ ORF followed by a TAA stop codon and a 303-bp 3′ untranslated region followed by a poly-A tail. The longest cDNA contains a 279-bp ORF encoding a 93-amino-acid sequence that is 50% identical to the C-terminal region of the *C. elegans* protein (Figure 4C).

l(3)3540 and l(3)6015: We also identified enhancer traps inserted into or very near to two prevously characterized genes on the third chromosome, *tramtrack* (ttk; Giesen *et al.* 1997) and *couch potato* (cpt; Bellen *et al.* 1992; Table 3). These two genes encode a DNA-binding and an RNA-binding protein respectively, both of which are involved in peripheral nervous system development.

l(2)2278: *PZ* homozygotes of *l(2)2278* developed normally until third instar. This last instar, however, was prolonged up to 8 days. Between days 4 and 8 of the extended instar, approximately 90% of the homozygotes pupariated but died before metamorphosing. Imaginal discs of these animals showed hyperplastic overgrowth, which might account for the prolonged third instar. Complementation experiments indicate that *l(2)2278* represents an allele of the *expanded* locus (Boedigheimer and Laughon 1993; data not shown).

l(2)4012: In this line the enhancer trap is inserted into a "1360" middle repetitive element (Kholodilov

et al. 1987) but there are no known transcriptional units close to the insertion. The mutation causes leg, wing, and bristle polarity defects and a behavioral phenotype suggestive of nervous system defects that cannot easily be attributed to an endocrine deficiency. The fact that the enhancer trap is inserted into a 1360 repetitive element has made any molecular characterization of this line difficult.

l(2)6072: Restriction enzyme analysis of the genomic DNA obtained by plasmid rescue from line *l(2)6072* indicated that the clone might contain concatamers of unrelated DNA. Sequence and PCR analyses showed that the first 308 bp of the clone does represent genomic DNA immediately adjacent to the *PZ* insertion, so this was used to screen a genomic library constructed from this line. A single genomic clone was isolated that contained most or all of the *PZ* insertion and 1.4 kb of flanking DNA. Sequencing confirmed that this 1.4-kb fragment was immediately adjacent to the insertion. Approximately 8×10^6 plaques of a wandering third-instar CNS/ring-gland cDNA library were screened using the 1.4-kb fragment as a probe, and two cDNA clones of 1.5 and 1.8 kb were plaque purified. The 5′ end of the longer cDNA has a 158-bp overlap with the 3′ end of the 308 bp adjacent to the *PZ* insertion, indicating that the insertion is 150 bp 5′ to the start of the longer cDNA. A BLAST analysis of the cDNA showed that it encodes the Drosophila homolog of the C subunit of V-ATPase and has high homology to tick, human, slime mold, and yeast C subunits (Figure 5). The conceptual translation of the Drosophila cDNA reported here is most homologous to the tick protein (Luo 1993).

Comparison of expression patterns of reporter *lacZ* as detected by X-Gal and antibody staining, and of nearby genes as detected by RNA in situ hybridization: We compared *lacZ* expression patterns in the ring gland, CNS, imaginal discs, lymph gland, fat body, and salivary glands of third-instar larvae of six lines, *l(2)1275*, *l(2)1857*, *l(2)2278*, *l(2)3909*, *l(2)4524*, and *l(2)6072*, as detected enzymatically using X-Gal and immunohistochemically using a monoclonal antibody to β-galactosidase. In most cases the overall staining patterns were very similar to each other although X-Gal staining was often more diffuse than antibody staining (Figure 3, A–D). This difference was most evident in lines such as *l(2)3909*, which show a dynamic expression pattern during the third instar (Figure 3, E–G). Within the ring gland, *lacZ*-positive LC were detected by both methods. However, in five of the fifteen cases where the MC also stained and in the three instances where the CCC also stained, expression in these non-LC ring gland cells was detected only immunohistochemically (Table 2; Figure 2, A and B). These discrepancies presumably reflect different sensitivities of the methods used.

Cloning of the genes affected by the *PZ* insertions made it possible to compare the expression of the reporter gene with that of the affected endogenous gene. We performed

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Fly     ---------MmSEYWIISAPGDKTCQQTYDTMNNLTSKQHNLCNNYKFHIPDLKVGTLDQLVGLSDDLGKLDTYVEQITR  71
Tick    -----------SEFWLISAPGEKTCQQTYEKLCSVTSKQNNLSTNYKFHLPDLKVGTLDQLVGLSDDLGKLDTYVEGVTR  69
Human   ----------MTEFWIISAPGEKTCQQTWEKLHAATSKNNNLAVTSKFNIPDLKVGTLDVLVGLSDELAKLDAFVEGVVK  70
Dictyo  -------MsETQEFWLISAPnlpgadi-FDQVNQKTAKENSLSENKKFNTPALRVGTlNSLITLNDElQKIDTIVESTTK  72
Yeast   matalytAndfilislpqnaqpvtapgsktdswfnetliggrAFVSDFKIPEFKIGSLDTLIVESEELSKVDNQIGASIG  80

Fly     KVANYLGEVLEDQRDKLHENLMANNTELPQYLTRFQWDMAKYPIKQSLRNIADIISKQIGQIDGDLKTKSQAYNNLKGNL  151
Tick    KVATYLGDVLDDQRDKLADNLLANGMEMEAYLTRFQWDMAKYPIKQSLKNITDIISKQVGQIDADLKSKSAAYNNLKTQL  149
Human   KVAQYMADVLEDSKDKVQENLLANGVDLVTYITRFQWDMAKYPIKQSLKNISEIIAKGVTQIDNDLKSRASAYNNLKGNL  150
Dictyo  KIARQLVD-LVGTKPGKDKSLSINGHTIPQYLQQFAWDDAKYNLKLSLQEIVEKISSAVSKIDDDLKIKSSEYSTLSSSV  151
Yeast   KIIEILQGLNETS-TNAYRTLPINNMPVPEYLENFQWQTRKFKLDKSIKDLITLSNESSQLDADVRATYANYNSAKTNL  159

Fly     QNLEKKKTGSLLTRNLADLVKKEHFILDSEYLTTLLVIVPKVMANDWLTNYEKITDMIVPRSSQLIQEDADYCLFNVTLF  231
Tick    QSIERKSTGSLLVRSLADLVRKEHFVLGSEYLITLLVVVPKMMYKDWYSKYEKLSDMVVPSSSQLVYEDNDHGLFTVTLF  229
Human   QNLERKNAGSLLTRSLAEIVKKDDFVLDSEYLVTLLVVVPKLNHNDWIKQYETLAEMVVPRSSNVLSEDQDSYLCNVTLF  230
Dictyo  ASEERKASGNLQVRTLNDLITA-DNIVQTDYFTTAFVVIPKQSEKEFLACYETISDFVLGRSAKRVAQDNDYFLYSVILF  230
Yeast   AAAERKKTGDLSVRSLHDIVKPEDFVLNSEHLTTVLVAVPKSLKSDFEKSYETLSKNVVPASASVIAEDAEYVLFNVHLF  239

Fly     KKVAEEFKLHARERKFIVRDFVYNEEELAAGKNEMPKLMTDKKKQFGPLVRWLKVNFSEAFCALIHVKALRVFVESVLRY  311
Tick    QKVVDTFKHHCRENKFVVRDFVYDESALLAGKNEIAKLESDKKKQYGLLVRWLKVNFSEAFTAWVHVKALRLFVESVLRY  309
Human   RKAVDDFRHKARENKFIVRDFQYNEEEMKADKEEMNRLSTDKKKQFGPLVRWLKVNFSEAFIAWIHVKALRVFVESVLRY  310
Dictyo  KKFYENFKTKIIEKKWVVRDFkl-Ednkptq--ErsKlTEDKKNCRTSLIRWCRLNFPEAFMAWVHLKVVRVFVESVLRF  307
Yeast   KKNVQEFTTAAREKKFIPREFNYSEELIDQLKKEHDSAASLEQSLRVQLVRLAKTAYVDVFINWFHIKALRVYVESVLRY  319

Fly     GLPVNFQAILIEPNKKSVKRLRDVLNQLYGHLDGASAGGAVSsadnvDIPGLGFGQSEYFPYVFYKVNIDMVEqakv-  388
Tick    GLPVNFQGMLLQPQKKTAKRLREVLNQLYSHLDTSIAQGPVD-----DIPGLNLGQQEYYPYVYFKISIDMADthvri  382
Human   GLPVNFQAMLLQPNKKTLKKLREVLHELYKHLDSSAAaiidapm---DIPGLNLSQQEYYPYVYYKIDCNLLEfk---  382
Dictyo  GIPFNFQAILMKPQKGADKKVRDILFDQFKYLGSAHisgknetddsekfypyisvsvnwen----------------  368
Yeast   GLPPHFNIKIIAVPPKNLSKCKSELIDAFGFLGGNAfmdkkgkinkqdtslhqyaslvdteyepfvmyiinl-----  392
```
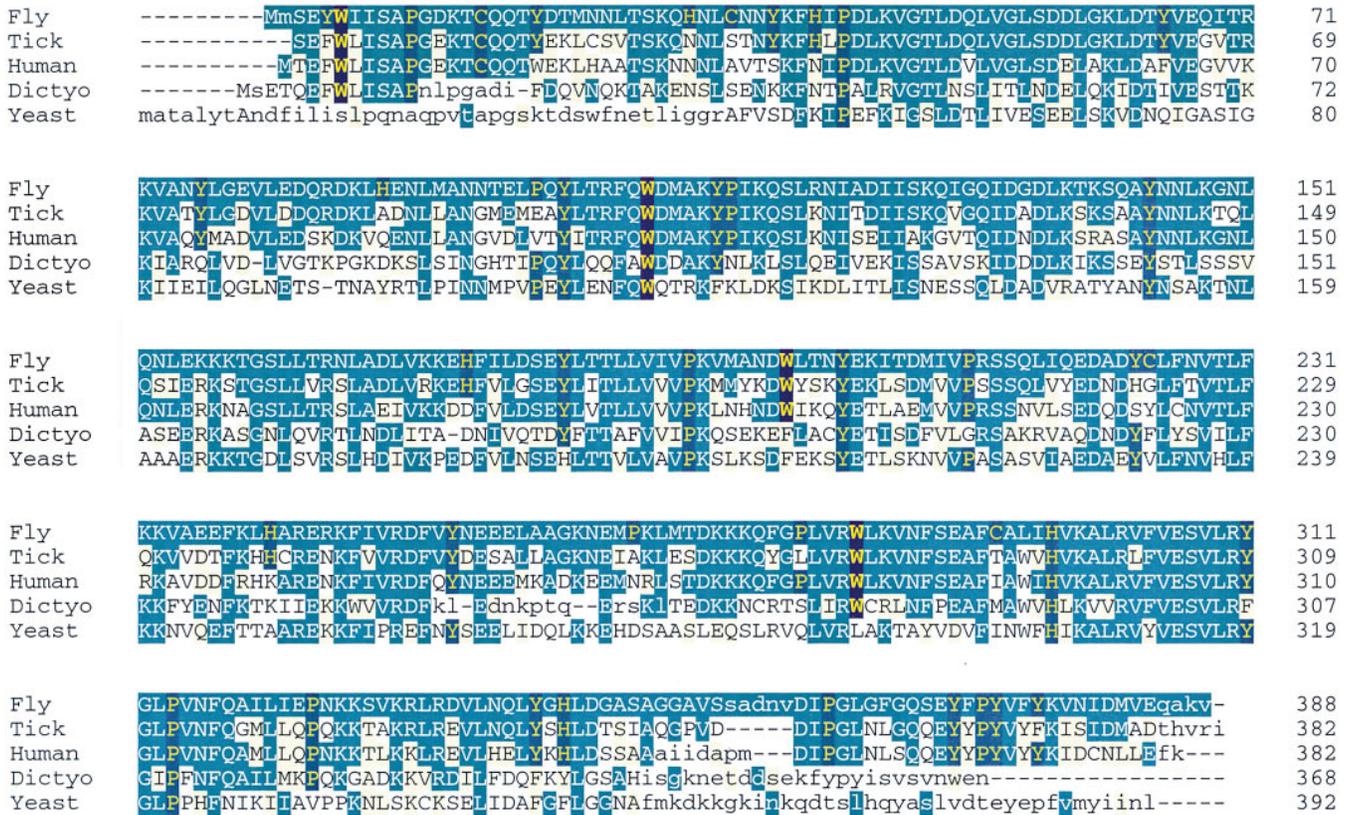
Figure 5.—Macaw alignments (Schuler *et al.* 1991) of the C subunits of V-ATPase from various sources. Darkness of shading indicates the significance of the match. References: fly, this study; tick (Luo 1993); Dictyostelium (Gerisch and Westphal, unpublished results), Genbank accession no. 1718089; yeast (Beltran *et al.* 1992); human (Van Hille *et al.* 1993).

RNA in situ hybridization on whole mounts of ring glands, CNS, imaginal discs, and lymph glands of heterozygous third-instar larvae of four lines, *l(2)1275*, *l(2)3909*, *l(2)4524*, and *l(2)6072*, using probes corresponding to transcribed regions of genes adjacent to the *PZ* insertions (Table 3). The probes used were a 539-bp PCR product from exon 2 of EF-1α F₁ (Hovemann *et al.* 1988), a 568-bp fragment of the calmodulin gene containing 3′ untranslated sequences common to both known transcripts (Kovalick and Beckingham 1992), a 920-bp genomic fragment containing coding and 3′ untranslated regions of a novel Drosophila gene, and a 1.8-kb cDNA clone for the Drosophila C subunit of vacuolar ATPase (V-ATPase), respectively. The RNA staining patterns for lines *l(2)1275*, *l(2)3909*, and *l(2)4524* closely matched the *lacZ* expression patterns detected by X-Gal and β-galactosidase antibody staining. For lines *l(2)1275*, *l(2)3909* and *l(2)4524* endogenous gene expression was detected in both the LC and the MC of the ring gland, which is in agreement with the antibody results noted above (see, for example, Figure 2, A–C). However, for line *l(2)6072*, the expression pattern for the V-ATPase C subunit gene was very different from the *lacZ* pattern. In third-instar larvae, reporter gene expression as assayed by X-Gal staining (Figure 2D), mAb to β-galactosidase (Figure 2E), or RNA *in situ* hybridization using a *lacZ* probe (Figure 2F) was limited to the MC of the ring gland. In contrast to these results, *in situ* hybridization using a probe for the C subunit gene revealed nearly ubiquitous expression in the ring gland (Figure 2G) as well as in the CNS, imaginal discs, and lymph gland. The expression pattern of the *lacZ* reporter gene differs significantly from that of other insertions and from that of an endogenous gene located less than 200 bp from the *PZ* insertion site.

DISCUSSION

We have identified 76 genes that are strongly expressed in the Drosophila ring gland during development. For nine of these, further studies of expression pattern, mutant phenotype and molecular nature identify the genes as strong candidates for playing an important role in endocrine functions controlling development. In some cases, the molecular nature of the predicted gene product is quite consistent with conclusions drawn from physiological studies of the endocrine system of larger insects. In other cases, our data suggest significant extension of present models for the regulation of insect endocrine glands.

Two of the genes we have identified encode products that have already been implicated in the functioning

of prothoracic glands of other insects. The insert in *l(2)3909* is 60 bp 5′ to the transcription start site of the calmodulin gene (Doyle *et al.* 1990). The gene is expressed exclusively and at high levels in the ring gland of third-instar larvae, suggesting an important, presumably endocrine function for calmodulin in that tissue as has already been suggested for lepidopterans (Gilbert *et al.* 1988; Granger *et al.* 1995). Calmodulin and other Ca^{2+}-binding proteins are integral to the transduction of a wide range of Ca^{2+}-dependent signals (Niki *et al.* 1996) and there is clear evidence for the Ca^{2+} dependence of EC production in the Manduca larval prothoracic gland (PTG), at least for the commitment peak early in the last larval instar. Studies of these glands *in vitro* show that changes in intracellular Ca^{2+} concentrations are both necessary and sufficient for the generation of the commitment peak of EC (Smith *et al.* 1986) and that PTTH-mediated stimulation of EC production requires extracellular Ca^{2+} (Rybcyznski and Gilbert 1994). Stimulation of EC production by brain extracts on isolated Drosophila ring glands is also Ca^{2+}-dependent (Henrich 1995). A simple interpretation is that binding of PTTH to its receptor initiates an influx of Ca^{2+} into the cell and that this influx activates downstream elements of the Ca^{2+}-cAMP-dependent signaling pathway. It is known that Ca^{2+} activates PTG adenylate cyclase both directly and as a complex when bound to calmodulin (Meller *et al.* 1990). Since cAMP phosphodiesterase activity is low at this stage (Smith and Pasquarello 1989), cAMP is expected to accumulate. Both large and small PTTH stimulate increased cAMP levels in PTG (Smith *et al.* 1984; Smith and Pasquarello 1989; Watson *et al.* 1993), and a rise in cAMP levels occurs with PTTH-stimulated EC production in early last-instar PTG.

The enhancer trap of *l(2)6353* is inserted into the 5′ untranslated region of the DC0 gene, which encodes the catalytic subunit of protein kinase A (PKA or cAMP-PK; Lane and Kalderon 1993). This protein probably functions downstream of cAMP in the Ca^{2+}-cAMP-dependent signaling pathway. PKA is activated in *M. sexta* PTGs by PTTH immediately prior to EC production (Smith *et al.* 1986). This is consistent with the idea that activation of the Ca^{2+}-cAMP-dependent signaling pathway by PTTH leads to PKA-dependent phosphorylation of key proteins including ribosomal protein S6 (Rountree *et al.* 1987; Combest and Gilbert 1992), and that this causes changes in selective translation leading to increased EC production.

The enhancer trap in *l(2)1275* is inserted 30 bp 5′ to the transcription start site of the gene encoding the translation elongation factor EF-1α F_1 (Hovemann *et al.* 1988). A role for this factor in hormone production and/or secretion has not been previously suggested, but it is plausible that it plays a role downstream of ribosomal protein S6 in the Ca^{2+}-cAMP-dependent signaling pathway. The EF-1α F2 gene is expressed at high levels

during metamorphosis (Walldorf *et al.* 1985), a time of higher and prolonged levels of EC (Hodgetts *et al.* 1977). Studies in *M. sexta* have shown that EC production is under translational control (Smith and Pasquarello 1989) and that certain proteins are selectively translated and phosphorylated in response to PTTH (Rybczynski and Gilbert 1994). This selective translation could result from the production and/or activation by phosphorylation (Venema *et al.* 1991) of EF-1α which has been shown to be a key regulator of translational control in other systems. Rapamycin, an inhibitor of S6 phosphorylation, dramatically inhibits selective translation of both EF-1α and EF-2 in mammalian cells (Terada *et al.* 1994), suggesting that synthesis of these elongation factors is selectively enhanced by S6 phosphorylation.

There is another possible function for EF-1α in the regulation of hormone titers. This factor is structurally conserved among diverse species including Drosophila and probably has similar functions in all organisms (Hovemann *et al.* 1988). In Tetrahymena, EF-1α has two entirely separate functions. In addition to its role in directing the binding of aminoacyl-tRNAs to the ribosome during translation, EF-1α can function as a Ca^{2+}/calmodulin-dependent F-actin bundling factor (Numata 1996). Changes in the actin cytoskeleton have been proposed to mediate neuropeptide and hormonal secretion (Trifaro and Vitale 1993). Ultrastructural studies have shown an increase in smooth endoplasmic reticulum and secretory vesicles throughout the final instar in EC-producing cells of both Drosophila ring glands (Aggarwal and King 1969) and Manduca PTG (Hanton *et al.* 1993). In flies, there is a 50-fold increase between 50 and 94 hr of development, followed by an additional 10-fold increase over the last 4 hr of the third instar. It is possible, therefore, that the hemolymph titer of EC is regulated both by biosynthetic rates and by control of secretion, and that EF-1α may be involved in regulating one or both of these processes.

The gene identified in *l(2)4524* and its *C. elegans* homolog do not show significant homology to any other known proteins. They do have some limited homology to a family of carboxylases, particularly to propionyl-CoA carboxylases, but it is not strong enough to support conjecture about function. However, the behavioral phenotype is very similar to that seen in *l(2)3909* and in weak *Cam* mutants.

We also identified enhancer traps inserted into or very near to two previously characterized genes on the third chromosome, *tramtrack* (*ttk*) and *couch potato* (*cpo*). Although these two genes are known from their roles in peripheral nervous system development (Bellen *et al.* 1992; Guo *et al.* 1995), it is likely that they have other functions as well. Amorphic *cpo* alleles are embryonic lethal, but the homozygous embryos show no obvious developmental abnormalities (Bellen *et al.* 1992). Ttk is required for embryonic glial cell development (Giesen *et al.* 1997) and it also functions in the assignment

of cell fates during sensory organ development (Xiong and Montell 1993; Guo *et al.* 1995). If expressed early enough, both of these genes could play roles in cell fate determination during ring gland development.

Complementation experiments indicate that *l(2)2278* represents an allele of the *expanded* locus, which controls wing size (Boedigheimer and Laughon 1993). The prolonged third instar observed in *l(2)2278* may be the result of the hyperplastic overgrowth of imaginal discs, rather than a direct effect of the mutation on ring gland function. Other imaginal disc overgrowth mutants also show prolonged larval life, apparently because overgrowing discs prevent the endocrine system from initiating metamorphosis, rather than because of a direct effect of the mutation on the endocrine system (Bryant and Levinson 1985). The significance of *expanded* expression in the ring gland is unknown at present. *l(2)4012* also shows leg, wing, and bristle abnormalities and a behavioral phenotype (uncoordinated movement, inability to fly) that cannot easily be attributed to any endocrine deficiency. The fact that the enhancer trap is inserted into a 1360 repetitive element has made any molecular characterization of this line difficult.

Our screen identified one enhancer trap, *l(2)6072*, with strong expression in the MC of the ring gland, which is thought to be the source of JH. Whether assayed for β-galactosidase activity with X-Gal or β-galactosidase protein production with a monoclonal antibody, the only significant *lacZ* reporter gene expression in *l(2)6072* larvae is in the MC during the second and third instars. With prolonged X-Gal incubation, low levels of expression are detected in the midgut and brain as well. *lacZ* expression increases in the pupal brain but in the ring gland it remains restricted to the MC. In adults, strong *lacZ* expression occurs in the rectal papillae.

The enhancer trap of *l(2)6072* is inserted 5′ to the coding sequence of the gene encoding the C subunit of V-ATPase (Van Hille *et al.* 1993). The main known function of V-ATPase in insects is to act as a proton pump to energize active transport at the apical plasma membrane of ion-transporting epithelia, for example the rectal papillae, midgut and malpighian tubules (Harvey 1992). That the reporter gene expression in *l(2)6072* represents the action of a legitimate C-subunit enhancer is supported by the strong *lacZ* expression seen in the rectal papillae. However, the specific expression of *lacZ* in the larval CA may represent a different C subunit/V-ATPase function in those cells during development. V-ATPases are known to play an important role in neurotransmission by providing the energy for the uptake of neurotransmitters into synaptic vesicles, and they may also be important in synaptic vesicle formation and in neurosecretion (Nelson 1993). It is therefore possible that the ring gland V-ATPase functions in the uptake of neuropeptides in the MC of the ring gland.

Since *lacZ* expression in this initial screen was detected by X-Gal staining, which often diffused through-

out the ring gland, it was not always evident for the remaining 75 ring gland-positive lines whether the EC-producing LC were the only ring gland cells expressing the reporter gene or whether the MCs and/or the CCC expressed *lacZ* as well. This issue was resolved for a subset of these lines by detecting beta-galactosidase immunohistochemically. In eight of nine instances where different ring gland patterns were observed with X-Gal and antibody staining, the antibody was more sensitive. In line *l(2)10280*, however, nuclear localization indicative of authentic reporter *lacZ* expression was detected in the LCs by X-Gal staining but antibody staining revealed only a light, diffuse staining pattern in the same cells.

Most ring gland-positive lines expressed *lacZ* in other tissues as well. The most common additional pattern was a uniform, usually diffuse staining of the optic lobes and ventral nerve cord, sometimes darker in the neuropile region of the optic lobes, that was observed in over half of the lines assayed including those lines that were ring gland-negative. This staining may represent, as suggested by Mlodzik and Hiromi (1992) a chronic pattern resulting from sequences contained within the *PZ* construct itself rather than from sequences in the flanking genomic DNA. Punctate staining in the CNS occurred at a much lower frequency and probably represents legitimate externally driven reporter-gene expression. The second most common additional *lacZ*-positive tissues were imaginal discs. Various combinations of disc types stained but usually the eye discs were included and often were the predominant or only discs to stain. Eye-disc staining typically occurred at and behind the morphogenetic furrow (Ready *et al.* 1976). This eye disc reporter gene activity has been noted previously for this and similar enhancer-trap constructs (Mlodzik and Hiromi 1992) and again may represent a chronic pattern resulting from sequences within the construct itself.

Another problem with enhancer-trap screening is that the reporter construct may detect only a subset of the regulatory elements controlling nearby genes. This is dramatically illustrated in line *l(2)6072* where, even though the enhancer trap is inserted within 150 bp of the 5′ end of the C subunit V-ATPase gene, *lacZ* is expressed almost exclusively in the MCs of the ring gland whereas the endogenous gene is ubiquitously expressed.

We have identified nine genes expressed in the ring gland during development and have suggested ways in which they could function in the neuroendocrine control of development. Quantification of hormone levels from these mutants as well as rescue experiments will be required to determine the precise nature of the endocrine defects, if any, in these mutants, and to define the normal functions of the set of gene products they identify.

LITERATURE CITED

Aggarwal, S. K., and R. C. King, 1969 A comparative study of the ring glands from wild type and *l(2)gl* mutant *Drosophila melanogaster*. J. Morphol. **129:** 171–200.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman, 1990 Basic local alignment search tool. J. Mol. Biol. **215:** 403–410.

Bellen, H. J., C. J. O'Kane, C. Wilson, U. Grossniklaus, R. K. Pearson *et al.*, 1989 P-element-mediated enhancer detection: a versatile method to study development in *Drosophila.* Genes Dev. **3:** 1288–1300.

Bellen, H. J., S. Kooyer, D. D'Evelyn and J. Pearlman, 1992 The *Drosophila* couch potato protein is expressed in nuclei of peripheral neuronal precursors and shows homology to RNA-binding proteins. Genes Dev. **6:** 2125–2136.

Bellen, H. J., H. Vaessin, E. Bier, A. Kolodkin, D. D'Evelyn *et al.*, 1992 The Drosophila couch potato gene: an essential gene required for normal adult behavior. Genetics **131:** 365–375.

Beltran, C., J. Kopecky, Y. C. Pan, H. Nelson and N. Nelson, 1992 Cloning and mutational analysis of the gene encoding subunit C of yeast vacuolar H(+)-ATPase. J. Biol. Chem. **267:** 774–779.

Bier, E., H. Vaessin, S. Shepherd, K. Lee, K. McCall *et al.*, 1989 Searching for pattern and mutation in the *Drosophila* genome with a P-lacZ vector. Genes Dev. **3:** 1273–1287.

Boedigheimer, M., and A. Laughon, 1993 Expanded: a gene involved in the control of cell proliferation in imaginal discs. Development **118:** 1291–1301.

Bryant, P. J., and P. Levinson, 1985 Intrinsic growth control in the imaginal primordia of Drosophila, and the autonomous action of a lethal mutation causing overgrowth. Dev. Biol. **107:** 355–363.

Cherbas, P., and L. Cherbas, 1996 Molecular aspects of ecdysteroid hormone action, pp. 175–221 in *Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells*, edited by L. I. Gilbert, J. R. Tata and B. G. Atkinson. Academic Press, San Diego.

Combest, W. L., and L. I. Gilbert, 1992 Polyamines modulate multiple protein phosphorylation pathways in the insect prothoracic gland. Mol. Cell. Endocrinol. **83:** 11–19.

Doyle, K. E., G. E. Kovalick, E. Lee and K. Beckingham, 1990 *Drosophila melanogaster* contains a single calmodulin gene. Further structure and expression studies. J. Mol. Biol. **213:** 599–605.

Giesen, K., T. Hummel, A. Stollewerk, S. Harrison, A. Travers *et al.*, 1997 Glial development in the Drosophila CNS requires concomitant activation of glial and repression of neuronal differentiation genes. Development **124:** 2307–2316.

Gilbert, L. I., W. L. Combest, W. A. Smith, V. H. Meller and D. B. Rountree, 1988 Neuropeptides, second messengers and insect molting. BioEssays **8:** 153–157.

Gilbert, L. I., R. Rybczynski and S. S. Tobe, 1996 Endocrine cascade in insect metamorphosis, pp. 60–107 in *Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells*, edited by L. I. Gilbert, J. R. Tata and B. G. Atkinson. Academic Press, San Diego.

Granger, N. A., L. G. Allen, S. L. Sturgis, W. Combest and R. Ebersohl, 1995 Corpora allata of the larval tobacco hornworm contain a calcium/calmodulin-sensitive adenylyl cyclase. Arch. Insect Biochem. Physiol. **30:** 149–164.

Grieneisen, M. L., 1994 Recent advances in our knowledge of ecdysteroid biosynthesis in insects and crustaceans. Insect Biochem. Mol. Biol. **24:** 115–132.

Grossniklaus, U., H. J. Bellen, C. Wilson and W. J. Gehring, 1989 P-element-mediated enhancer detection applied to the study of oogenesis in Drosophila. Development **107:** 189–200.

Guo, M., E. Bier, L. Y. Jan and Y. N. Jan, 1995 Tramtrack acts downstream of numb to specify distinct daughter cell fates during asymmetric cell divisions in the Drosophila PNS. Neuron **14:** 913–925.

Hanton, W. K., R. D. Watson and W. E. Bollenbacher, 1993 Ultrastructure of prothoracic glands during larval-pupal development of the tobacco hornworm, *Manduca sexta*: a reappraisal. J. Morphol. **216:** 95–112.

Harvey, W. R., 1992 Physiology of V-ATPases. J. Exp. Biol. **172:** 1–17.

Heiman, R. G., R. C. Atkinson, B. F. Andruss, C. Bolduc, G. E. Kovalick *et al.*, 1996 Spontaneous avoidance behavior in Drosophila null for calmodulin expression. Proc. Natl. Acad. Sci. USA **93:** 2420–2425.

Henrich, V. C., 1995 Comparison of ecdysteroid production in *Drosophila* and *Manduca*: pharmacology and cross-species neural reactivity. Arch. Insect Biochem. Physiol. **30:** 239–254.

Henrich, V. C., M. D. Pak and L. I. Gilbert, 1987 Neural factors that stimulate ecdysteroid synthesis by the larval ring gland of *Drosophila melanogaster*. J. Comp. Physiol. Biochem. Syst. Environ. **157:** 543–549.

Hodgetts, R. B., B. Sage and J. D. O'Connor, 1977 Ecdysone titers during postembryonic development of *Drosophila melanogaster*. Dev. Biol. **60:** 310–317.

Horowitz, H., and C. A. Berg, 1995 Aberrant splicing and transcription termination caused by *P* element insertion into the intron of a Drosophila gene. Genetics **139:** 327–335.

Hovemann, B., S. Richter, U. Walldorf and C. Cziepluch, 1988 Two genes encode related cytoplasmic elongation factors 1 alpha (EF-1 alpha) in Drosophila melanogaster with continuous and stage specific expression. Nucleic Acids Res. **16:** 3175–3194.

Innis, M. A., D. H. Gelfand, J. J. Shinsky and T. J. White, 1990 *PCR Protocols: A Guide to Methods and Applications.* Academic Press, New York.

Kalderon, D., and G. M. Rubin, 1988 Isolation and characterization of *Drosophila* cAMP-dependent protein kinase genes. Genes Dev. **2:** 1539–1556.

Karpen, G. H., and A. C. Spradling, 1992 Analysis of subtelomeric heterochromatin in the Drosophila minichromosome Dp1187 by single *P* element insertional mutagenesis. Genetics **132:** 737–753.

Kholodilov, N. G., V. N. Bol'shakov, V. M. Blinov, V. V. Solov'ev and I. F. Zhimulev, 1987 Molecular-genetic characteristics of a new element in the Drosophila melanogaster genome with variable localization. Dokl. Akad. Nauk. SSSR. **295:** 984–989.

Kim, A. J., G. H. Cha, K. Kim, L. I. Gilbert and C. C. Lee, 1997 Purification and characterization of the prothoracicotropic hormone of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA **94:** 1130–1135.

King, R. C., S. K. Aggarwal and D. Bodenstein, 1966 The comparative submicroscopic morphology of the ring gland of *Drosophila melanogaster* during the second and third larval instars. Z. Zellforsch. Mikrosk. Anat. **73:** 272–285.

Kovalick, G. E., and K. Beckingham, 1992 Calmodulin transcription is limited to the nervous system during *Drosophila* embryogenesis. Dev. Biol. **150:** 33–46.

Lane, M. E., and D. Kalderon, 1993 Genetic investigation of cAMP-dependent protein kinase function in Drosophila development. Genes Dev. **7:** 1229–1243.

Luo, C., 1993 Molecular biology approach to investigate enzymes in tick gland which are important in tick feeding. Ph.D. Thesis/Dissertation, Oklahoma State University, Stillwater, OK.

Meller, V., S. Sakurai and L. I. Gilbert, 1990 Developmental regulation of calmodulin-dependent adenylate cyclase activity in an insect endocrine gland. Cell Regul. **1:** 771–780.

Mizoguchi, A., H. Ishizaki, H. Nagasawa, H. Kataoka, A. Isogai *et al.*, 1987 A monoclonal antibody against a synthetic fragment of bombyxin (4K-prothoracicotropic hormone) from the silkmoth, *Bombyx mori*: characterization and immunohistochemistry. Mol. Cell. Endocrinol. **51:** 227–235.

Mlodzik, M., and Y. Hiromi, 1992 Enhancer trap method in *Drosophila:* its application to neurobiology, pp. 397–414 in *Methods in Neurosciences, Vol. 9: Gene Expression in Neural Tissues*, edited by P. M. Conn. Academic Press, New York.

Mount, S. M., C. Burks, G. Hertz, G. D. Stormo, O. White *et al.*, 1992 Splicing signals in *Drosophila:* intron size, information content, and consensus sequences. Nucleic Acids Res. **20:** 4255–4262.

Nelson, N., 1993 Presynaptic events involved in neurotransmission. J. Physiol. (Paris) **87:** 171–178.

Niki, I., H. Yokokura, T. Sudo, M. Kato and H. Hidaka, 1996 Ca^{2+} signaling and intracellular Ca^{2+} binding proteins. J. Biochem. (Tokyo) **120:** 685–698.

Numata, O., 1996 Multifunctional proteins in Tetrahymena: 14-nm filament protein/citrate synthase and translation elongation factor-1 alpha. Int. Rev. Cytol. **164:** 1–35.

O'Kane, C. J., and W. J. Gehring, 1987 Detection in situ of genomic

regulatory elements in Drosophila. Proc. Natl. Acad. Sci. USA **84:** 9123–9127.

Pak, J. W., K. W. Chung, C. C. Lee, K. Kim, Y. Namkoong *et al.*, 1992 Evidence for multiple forms of the prothoracicotropic hormone in *Drosophila melanogaster* and indication of a new function. J. Insect Physiol. **38:** 167–176.

Pirrotta, V., 1986 Cloning *Drosophila* genes, pp. 83–110 in *Drosophila: A Practical Approach*, edited by D. B. Roberts. IRL Press, Oxford.

Ready, D. F., T. E. Hanson and S. Benzer, 1976 Development of the *Drosophila* retina, a neurocrystalline lattice. Dev. Biol. **53:** 217–240.

Reagan, J. D., W. H. Miller and S. J. Kramer, 1992 Allatotropin-induced formation of inositol phosphates in the corpora allata of the moth, *Manduca sexta.* Arch. Insect Biochem. Physiol. **20:** 145–155.

Rees, H. H., 1985 Biosynthesis of Ecdysone, pp. 249–293 in *Comprehensive Insect Biochemistry, Physiology and Pharmacology. Volume 7: Endocrinology I*, edited by G. A. Kerkut and L. I. Gilbert. Pergamon Press, New York.

Richard, D. S., S. W. Applebaum, T. J. Sliter, F. C. Baker, D. A. Schooley *et al.*, 1989 Juvenile hormone bisepoxide biosynthesis *in vitro* by the ring gland of *Drosophila melanogaster*: a putative juvenile hormone in the higher Diptera. Proc. Natl. Acad. Sci. USA **86:** 1421–1425.

Richard, D. S., S. W. Applebaum and L. I. Gilbert, 1990 Allatostatic regulation of juvenile hormone production in vitro by the ring gland of Drosophila melanogaster. Mol. Cell. Endocrinol. **68:** 153–161.

Riddiford, L. M., 1978 Ecdysone-induced change in cellular commitment of the epidermis of the tobacco hornworm, Manduca sexta, at the initiation of metamorphosis. Gen. Comp. Endocrinol. **34:** 438–446.

Roberts, B., L. I. Gilbert and W. E. Bollenbacher, 1984 *In vitro* activity of dipteran ring glands and activation by the prothoracicotropic hormone. Gen. Comp. Endocrinol. **54:** 469–477.

Rountree, D. B., W. L. Combest and L. I. Gilbert, 1987 Protein phosphorylation in the prothoracic glands as a cellular model for juvenile hormone-prothoracicotropic hormone interactions. Insect Biochem. **17:** 943–948.

Rybczynski, R., and L. I. Gilbert, 1994 Changes in general and specific protein synthesis that accompany ecdysteroid synthesis in stimulated prothoracic glands of *Manduca sexta.* Insect Biochem. Mol. Biol. **24:** 175–189.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schuler, G. D., S. F. Altschul and D. J. Lipman, 1991 A workbench for multiple alignment construction and analysis. Proteins Struct. Funct. Genet. **9:** 180–190.

Smith, W. A., 1995 Regulation and consequences of cellular changes in the prothoracic glands of *Manduca sexta* during the last larval instar: a review. Arch. Insect Biochem. Physiol. **30:** 271–293.

Smith, W. A., L. I. Gilbert and W. E. Bollenbacher, 1984 The role of cyclic AMP in the regulation of ecdysone synthesis. Mol. Cell. Endocrinol. **37:** 285–294.

Smith, W. A., W. L. Combest and L. I. Gilbert, 1986 Involvement of cyclic AMP-dependent protein kinase in prothoracicotropic hormone-stimulated ecdysone synthesis. Mol. Cell. Endocrinol. **47:** 25–33.

Smith, W. A., and T. J. Pasquarello, 1989 Developmental changes in phosphodiesterase activity and hormonal response in the prothoracic glands of Manduca sexta. Mol. Cell. Endocrinol. **63:** 239–246.

Tautz, D., and C. Pfeifle, 1989 A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback.* Chromosoma **98:** 81–85.

Terada, N., H. R. Patel, K. Takase, K. Kohno, A. C. Nairn *et al.*, 1994 Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. Proc. Natl. Acad. Sci. USA **91:** 11477–11481.

Trifaro, J. M., and M. L. Vitale, 1993 Cytoskeleton dynamics during neurotransmitter release. Trends Neurosci. **16:** 466–472.

Van Hille, B., M. Vanek, H. Richener, J. R. Green and G. Bilbe, 1993 Cloning and tissue distribution of subunits C, D, and E of the human vacuolar H(+)-ATPase. Biochem. Biophys. Res. Commun. **197:** 15–21.

Venema, R. C., H. I. Peters and J. A. Traugh, 1991 Phosphorylation of elongation factor 1 (EF-1) and valyl-tRNA synthetase by protein kinase C and stimulation of EF-1 activity. J. Biol. Chem. **266:** 12574–12580.

Walldorf, U., B. Hovemann and E. K. F. Bautz, 1985 *F1* and *F2*: two similar genes regulated differently during development of *Drosophila melanogaster.* Proc. Natl. Acad. Sci. USA **82:** 5795–5799.

Watson, R. D., W. E. Yeh, D. P. Muehleisen, C. J. Watson and W. E. Bollenbacher, 1993 Stimulation of ecdysteroidogenesis by small prothoracicotropic hormone: role of cyclic AMP. Mol. Cell. Endocrinol. **92:** 221–228.

Wilson, C., R. K. Pearson, H. J. Bellen, C. J. O'Kane, U. Grossniklaus *et al.*, 1989 P-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila.* Genes Dev. **3:** 1301–1313.

Wilson, R., R. Ainscough, K. Anderson, C. Baynes, M. Berks *et al.*, 1994 2.2 Mb of contiguous nucleotide sequence from chromosome III of C. elegans. Nature **368:** 32–38.

Xiong, W.-C., and C. Montell, 1993 *tramtrack* is a transcriptional repressor required for cell fate determination in the *Drosophila* eye. Genes Dev. **7:** 1085–1096.

Zitnan, D., F. Sehnal and P. J. Bryant, 1993 Neurons producing specific neuropeptides in the central nervous system of normal and puparation-delayed Drosophila. Dev. Biol. **156:** 117–135.