Developmental Expression of the Glucose Dehydrogenase Gene in *Drosophila melanogaster*

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ABSTRACT

The *Gld* gene of *Drosophila melanogaster* is transiently expressed during every stage of development. The temporal pattern of *Gld* expression is highly correlated with that of ecdysteroids. Exogeneous treatment of third instar larvae with 20-hydroxyecdysone induces the accumulation of *Gld* mRNA in the hypoderm and anterior spiracular gland cells. During metamorphosis *Gld* is expressed in a variety of tissues derived from the ectoderm. In the developing reproductive tract, *Gld* mRNA accumulates in the female spermathecae and oviduct and in the male ejaculatory duct and ejaculatory bulb. These four organs are derived from closely related cell lineages in the genital imaginal disc. Since the expression of *Gld* is not required for the development of these reproductive structures, this spatial pattern of expression is most likely a fortuitous consequence of a shared regulatory factor in this cell lineage. At the adult stage a high level of the *Gld* mRNA is only observed in the male ejaculatory duct.

SEVERAL developmentally regulated genes are currently being studied to elucidate the mechanisms underlying cellular differentiation. Such genes provide entry points into the regulatory circuits of development. The *Drosophila Glucose dehydrogenase* gene (*Gld*) exhibits a diverse array of developmental properties which make it a useful probe for investigating multiple regulatory circuits (CAVENER and MACINTYRE 1983; CAVENER 1985; CAVENER *et al.* 1986, CAVENER 1987; MURTHA and CAVENER 1989). The essential function of GLD enzyme is defined by null mutants which are unable to eclose due to defects in the formation of the operculum during pupariation (CAVENER, OTTESON and KAUFMAN 1986). *Gld* mRNA was previously detected in the integument of wandering third instar larvae and in the ejaculatory ducts of adult males after eclosion (CAVENER *et al.* 1986). Furthermore, the expression of the 2.8-kb *Gld* RNA can be prematurely induced in third instar larvae by 20-hydroxyecdysone (β-ecdysone) (MURTHA and CAVENER 1989). In order to examine the temporal relationship between *Gld* mRNA and ecdysteroids, a detailed analysis of the temporal regulation of *Gld* was undertaken. In addition the spatial pattern of *Gld* mRNA expression was examined in embryos, third instar larvae, pharate adults, and adults. Inasmuch as several features of these data were not predicted from the *Gld* mutant phenotype, we suggest that an exhaustive analysis of developmental expression is a prerequisite for an accurate assessment of regulatory controls and their evolution.

MATERIALS AND METHODS

Northern blot analysis: RNA samples were obtained from the Oregon-R strain of *D. melanogaster* reared on a standard cornmeal-molasses-yeast diet at 25°. RNA was extracted (Figure 1) every 2–4 hr during embryogenesis and the three larval instars and during every morphological stage of prepupal, pupal and pharate adult development as defined by BAINBRIDGE and BOWNES (1981). The developmental stages of the embryos were also confirmed through microscopic observation. The last time point sampled in the third larval instar (Figure 1, 44W) occurred less than 2 hr before the white prepupal stage (Figure 1, P1). The approximate mid-point of the 15 metamorphic stages (BAINBRIDGE and BOWNES 1988) are as follows (time zero at 25° set at pupariation): P1–0.15 hr (white prepupa), P2–0.85 hr (brown prepupa), P3–4.4 hr (bubble prepupa), P4–9.5 hr (cryptocephalic pupa), P5–15.4 hr (phanerocephalic pupa), P6–25.6 hr (pupa), P7–38.3 hr (pupa), P8–50.4 hr (yellow eye-pharate adult), P9–65.6 hr (amber eye-pharate adult), P10–74.6 hr (red eye-pharate adult), P11–76.0 hr (thoracic bristle-pharate adult), P12–78 hr (gray wing tips-pharate adult), P13–80 hr (gray wings-pharate adult), P14–87 hr (mature bristles-pharate adult), P15–96 hr (green meconium-pharate adult). After adult eclosion, RNA was extracted from virgin females and males at several time points through 30 hr after eclosion (Figure 1, bottom).

Total RNA was fractionated by electrophoresis on formaldehyde-agarose gels using previously published procedures (CAVENER *et al.* 1986). Northern blots on Hybond-N (Amersham) were hybridized with ^{32}P-cRNA transcribed from pSG1a which contains the major portion of the *Gld* coding region. Hybridizations were conducted in 50% formamide at 58° for 18 hr. After hybridization the filters were

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washed at room temperature with 2 × SSC-0.2% SDS for 30 min and then at 65° with 0.2 × SSC-0.2% SDS for 1 hr. Each of these developmental profiles were repeated at least once in independent experiments using independently isolated RNA samples. The Northern filters were also reprobed with cRNA probes for other genes (*rp49*, *sas* and/or *ted*) to confirm the integrity and relative abundance of the RNA samples.

***In situ* hybridization:** Embryos were staged by time after oviposition and fixed according to the procedure of MITCHISON and SADAT (1983). Wandering third-instar larvae and staged pharate adults (P7–9) (BAINBRIDGE and BOWNES 1981) were fixed either in Bouin-Duboscq fixative [WHITE and KANKEL (1978) and M. KIMBLE personal communication] or 4% paraformaldehyde/0.1 glutaraldehyde in PBS, pH 7.0. The pupae were removed from the puparium before embedding in paraffin. Sections were prepared and hybridized with hydrolyzed cRNA probes (HAFEN *et al.* 1983; INGHAM, HOWARD and ISH-HOROWICZ 1985; LYNN *et al.* 1983). *Gld* ^{35}S cRNA probes were made from pSG9a (CAVENER *et al.* 1986). Emulsion (Kodak NTB-2) coated sections were exposed for 7 days, developed, stained with hematoxylin/eosin and mounted with permount. Negative controls consisted of pretreating representative sections with RNAse prior to hybridization and also hybridization with cRNA probes made against vector alone or from plasmids containing other Drosophila genes. The hybridization patterns reported herein have been observed on duplicate slides from independent experiments.

Whole organ hybridizations were performed on the adult male reproductive tract using the methods of KORNBERG and co-workers (1985). After hybridization the organs were adhered to a slide with gelatin, dehydrated, dried, and coated with emulsion. After exposure for 7 days, the slide was developed and stained with hematoxylin.

RESULTS

Temporal expression of *Gld* mRNA: Northern analysis of 73 time points spanning the Drosophila developmental cycle through 30 hr post-eclosion was performed for *Gld* mRNA. *Gld* mRNA is expressed transiently during every major developmental stage (embryos, the larval instars, pupae and pharate adults) before becoming predominately male-limited at the adult stage (Figure 1). A comparison of the *Gld* RNA developmental profile (Figure 1) with that of the ecdysteroid profile of fly homogenates (Figure 2) revealed a striking correlation. To a large extent the changes in the level of *Gld* RNA parallels the changes in ecdysteroid levels. Changes in *Gld* mRNA typically follow changes in ecdysteroid by a few hours. This pattern is similar, although not identical, to that seen for dopa decarboxylase mRNA which is also regulated by ecdysteroids (MARSH and WRIGHT 1980; HIRSCH, MORGAN and SCHOLNICK 1986).

A low level of *Gld* RNA is first observed in 10–12-hr embryos, reaching a peak between 14 and 18 hr, followed by a decline to nondetectable levels at the end of embryogenesis (21–24 hr) (Figure 1, top). During the first two larval instars, *Gld* mRNA is expressed at peak levels from 14 to 18 hr in first instar larvae (Figure 1, 1st instar) and 12 to 20 hr in second instar larvae (Figure 1, 2nd instar). These two peaks in expression follow ecdysteroid peaks (Figure 2). A gradual increase in *Gld* transcript level is seen during feeding in the third instar larvae (Figure 1, 3rd instar). A sudden increase in transcript level occurs within a few hours before pupariation. This increase closely parallels a dramatic increase in ecdysteroids.

The level of *Gld* transcript decreases very quickly within the first few hours after pupariation (P1). During the prepupal stages (P1–3) and the first pupal stage (P4) the anterior spiracles are everted and the larval cuticle is modified into a puparial case most likely by the action enzymes secreted into the molting fluid (POODRY 1980; RIDDIFORD 1985). It is at this point that the operculum of the puparium is formed. As described below, GLD activity has been detected in the presumptive molting fluid and in the anterior spiracular gland cells at this time; in addition, the overall level of enzyme activity is seen to rise (CAVENER *et al.* 1986; P. GUNARATNE and D. CAVENER, unpublished data).

A large increase in *Gld* transcript is detectable at P6 (corresponding to approximately 144 hr in Figure 2) followed by a sustained high plateau (stages P8–12) and finally a rapid decline immediately before eclosion (stages P13–15). Although the accumulation of *Gld* mRNA during this period closely parallels the increase in ecdysteroid levels, a high level of *Gld* mRNA persists for approximately 10 hr beyond the rapid decline in ecdysteroids. (The 180 hr time point in Figure 2 corresponds to stage P9 in Figure 1.) The development of the adult is completed at the end of P15, when the adult ecloses (*i.e.*, emerges) from the puparium. At this point *Gld* RNA has fallen to a very low level.

Immediately after eclosion, *Gld* RNA is nearly undetectable in both sexes and then begins to rise rapidly in adult males reaching a plateau around 24 hr post-eclosion (Figure 1, bottom). Shortly after 24 hr, male GLD enzyme activity and mRNA reach peak levels coincident with sexual maturation (CAVENER *et al.* 1986). GLD enzyme and mRNA remain very low in females after eclosion at a level equivalent to that of P14 pharate adults.

Spatial expression of *Gld* mRNA: In stage 17 embryos (corresponding to approximately 18 hr in Figure 1, top), *Gld* expression is seen in three distinct regions (Figure 3). One of these sites corresponds to the hypophysis and the tissue surrounding the salivary duct. Sections not shown indicate that the *Gld* mRNA is not expressed in the salivary glands or duct. In the gnathal segments, signal is detected in the mandibular-maxillary regions. In addition to neuronal cells, these developing sensory organs have epidermal cells which secrete cuticular components (CAMPOS-ORTEGA and HARTENSTEIN 1985). In the posterior of the embryo,

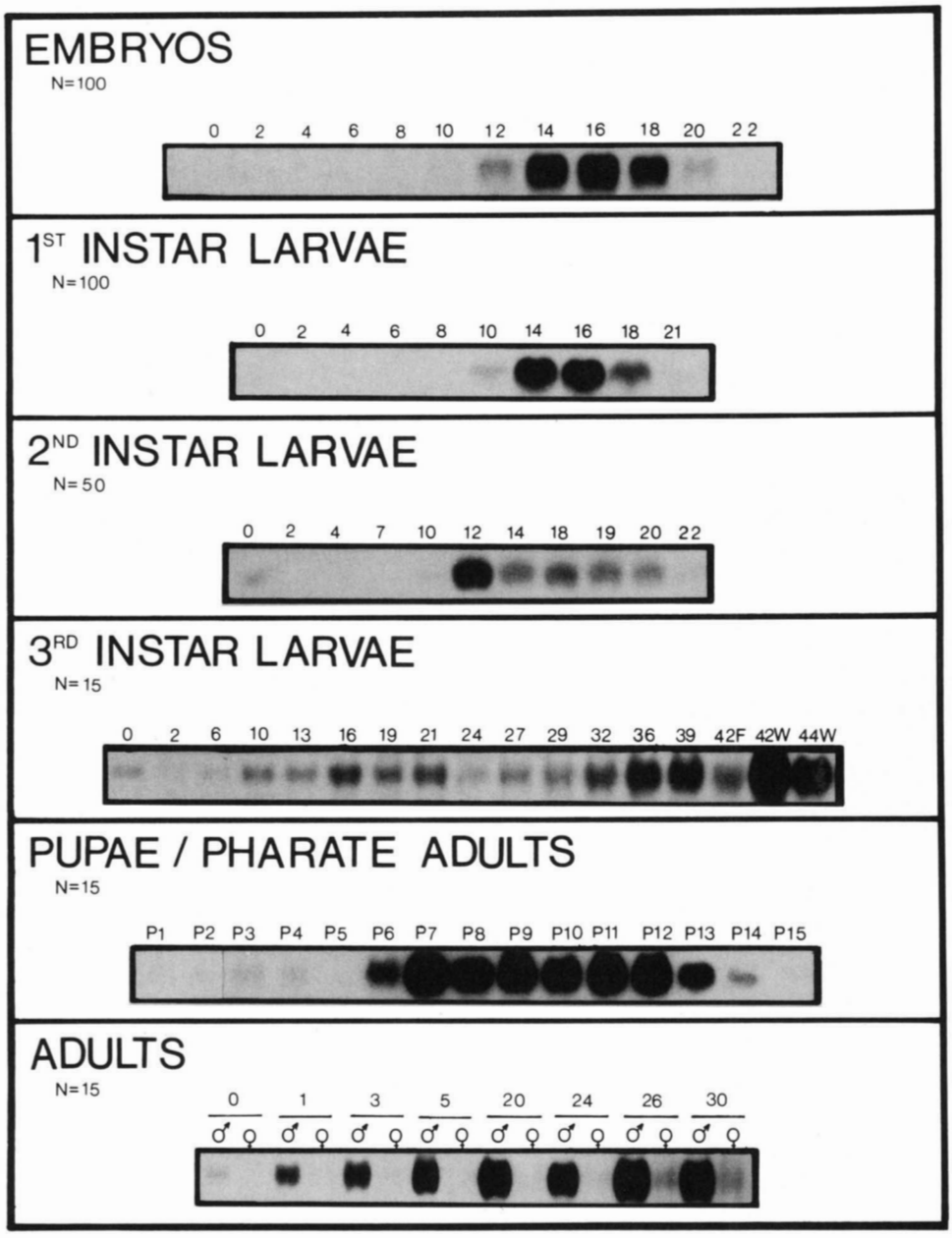

FIGURE 1.—Northern analysis of *Gld* 2.8-kb mRNA expression in embryos, first, second and third instar larvae, pupae/pharate adults, and adults. N = number of individuals from which total RNA was extracted and subjected to Northern analysis for each stage. The age in hours is given above each lane except for the prepupae, pupae and pharate adults. F = feeding, late third instar larvae. W = wandering, late third instar larve. Prepupae, pupae and pharate adults were morphologically staged using the method of BAINBRIDGE and BOWNES (1981). Each stage was examined in at least two experiments using independently isolated RNA samples. Northern blots were reprobed for the expression of one or more of the following genes: *rp49*, *sas* and *ted* to confirm the integrity of the RNA samples (not shown).

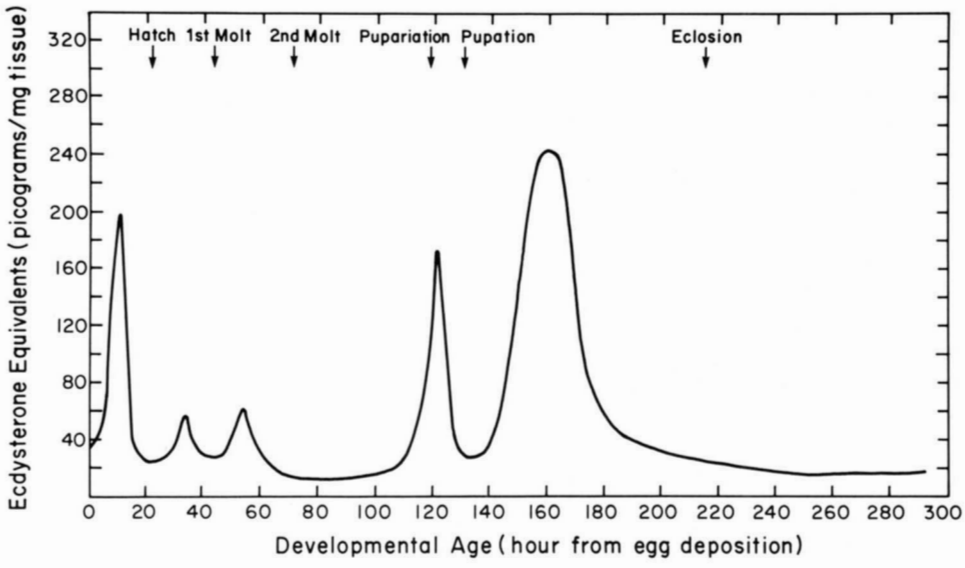

FIGURE 2.—Ecdysterone (β-ecdysone) developmental profile. Data provided by ROSS HODGETTS as shown in Figure 1 of KRAMINSKY *et al.* (1980).

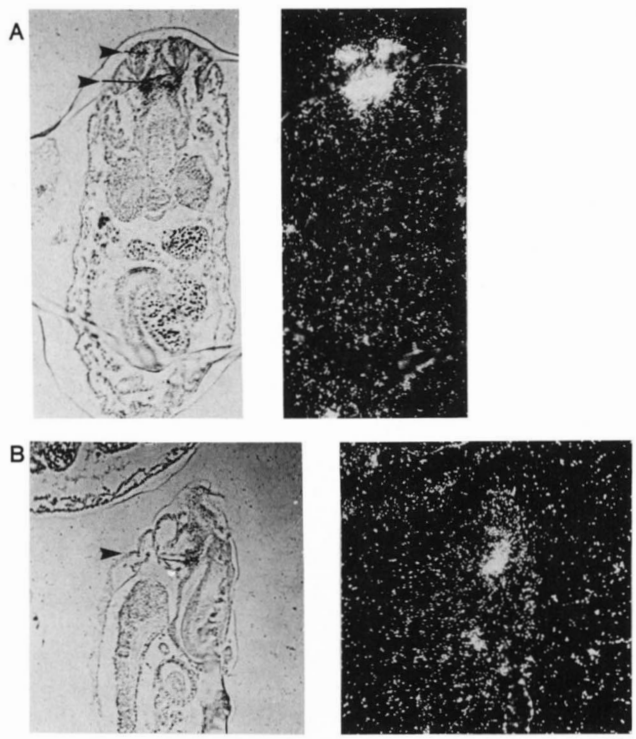

FIGURE 3.—*In situ* localization of *Gld* mRNA in stage 17 embryos. (A) Frontal section showing hybridization signal over the antennal-maxillary complex (upper arrow) and the hypophysis (lower arrowhead). (B) Sagittal section showing hybridization signal over the hypophysis ventral to the pharnyx.

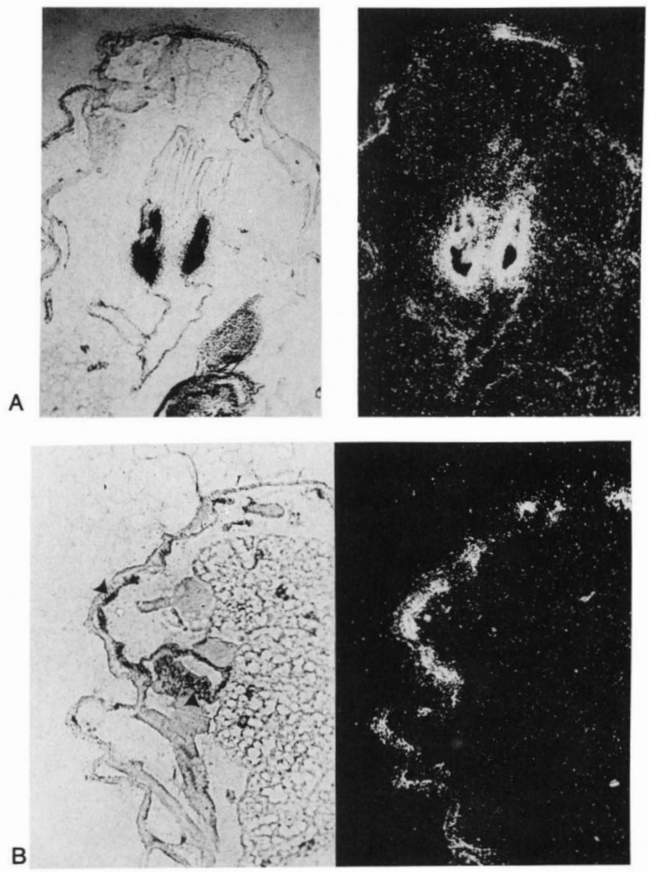

FIGURE 4.—*In situ* localization of *Gld* mRNA in late third instar larvae. (A) Frontal section showing intense hybridization signal over the anterior spiracular gland cells and the epidermal cells in the hypoderm. (B) Posterior sagittal section showing hybridization signal over the epidermal cells in the hypoderm (arrow top-left). Note the absence of signal over the genital imaginal disc (arrow in the middle of the figure).

expression is seen near an undefined tissue immediately adjacent to the posterior spiracles. There is no expression in the hypodermis at this stage in contrast to third instar larvae (see below).

Prior to stage 17, *Gld* RNA is observed in the clypeolabrum and in the mandibular-maxillary regions of the head (stage 14–17) and in the cells surrounding the posterior spiracles (data not shown). The expression in the gnathal segments coincides with the location of two sensory organs: the hypopharyngeal/labial complex (hypophysis and epiphysis) and the antennal-maxillary complex. [See POULSON (1950) and CAMPOS-ORTEGA and HARTENSTEIN (1985) for a description of these tissues.] In stage 15 embryos the message is restricted to the anterior end of the stomadeum near the location of the hypophysis and epiphysis. Head involution carries these regions from the anterior end of the embryo at stage 14 to the interior at stage 17.

In the wandering third instar larva, the tissue specific expression of *Gld* is profoundly different from the pattern observed in embryos. *Gld* RNA is detected in the larval epidermal cells lining the external cuticle and is abundantly expressed the anterior spiracular glands: a cluster of large, polyploid gland cells found at the base of each of the anterior lateral spiracles (Figure 4). [See WHITTEN (1980, p. 507) for a description of these cells.] Although these two tissues display temporal differences in their expression during the third larval instar, both exhibit induction of *Gld* RNA shortly after treatment with 20-hydroxyecdysone (Figure 5) as was previously demonstrated for whole larvae (MURTHA and CAVENER 1989).

Gld mRNA expression drops abruptly within the first hour after the onset of pupariation during the P1 stage (Figure 1), although moderate levels of GLD enzyme activity persist throughout the pupal period (CAVENER *et al.* 1986). During pupariation (P1–4), the anterior spiracles become everted and the anterior seam of the operculum is formed (BAINBRIDGE and BOWNES 1981). During the P4 stage the larval epidermis pulls away from the old larval cuticle at the anterior spiracles, secreting molting fluid in the region of operculum formation (Figure 6). We speculate that the gland cells at the base of the spiracles secrete molting fluid into the exuvial space during the larval-pupal molt and that one of its essential functions is to modify the operculum seams in preparation for eclosion. During the P4 stage of development, presumptive molting fluid was isolated from the operculum

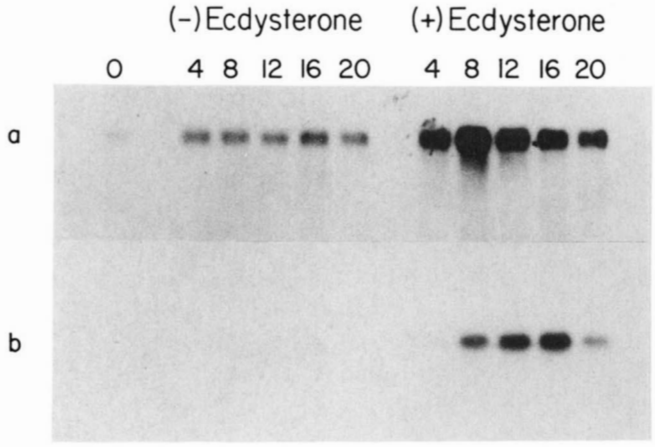

FIGURE 5.—20-Hydroxyecdysone induction of *Gld* mRNA expression in anterior spiracular glands and hypoderm. OreR larvae were reared at 25° until 65 hr after hatching (time zero), and then fed a solution containing 1 mM 20-hydroxyecdysone (+) or a control solution lacking hormone (−) for up to 20 hr. These animals were then bisected just posterior to the spiracular glands, and RNA isolated for Northern analysis. Sample (a) contains the anterior most segments enriched for the spiracular glands but containing some hypodermal cells. Sample (b) contains posterior segments (approximately 80% of the larva). The indicated time points are hours after hormone administration.

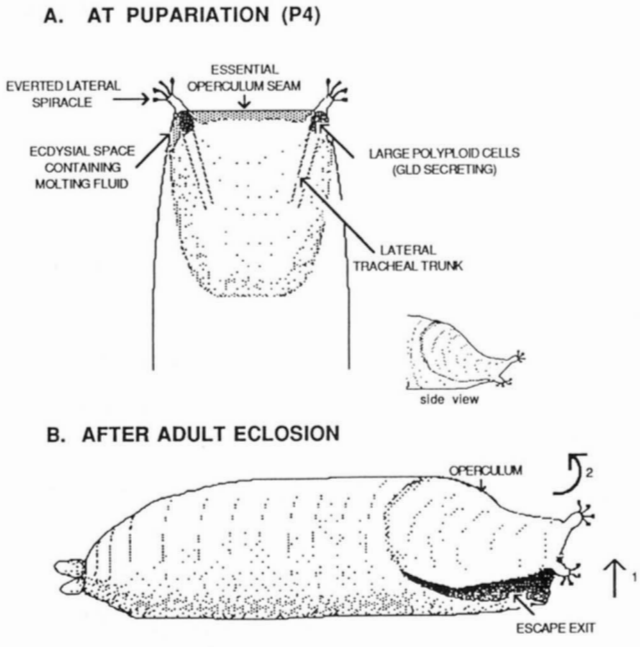

FIGURE 6.—Diagrammatic representation of the formation of the operculum. (A) Dorsal view during apolysis at pupariation (stage P4); inset shows side view of puparium. (B) Oblique view of puparium with operculum after adult eclosion. Numbered arrows indicate the sequence of the opening of the puparium with the front seam of the operculum breaking first, followed by tearing of the lateral seams and lifting of the operculum. The adult fly then crawls out through the opening.

region and found to contain GLD activity. A thin, capillary needle was used to pierce the cuticle near the edges of the lateral spiracles and inserted into the exuvial space where cells had pulled away from the old larval cuticle. This fluid was collected from five P4 individuals, electrophoresed on native gels, and stained for GLD. The presumptive molting fluid exhibited two bands of GLD activity with identical mobility as the GLD electromorphs from whole body extracts (data not shown).

The rest of the puparium is also modified at pupariation, apparently by molting fluid enzymes secreted by the epidermis. In contrast to the resilient and flexible puparia of *Gld* null mutants, wild-type puparia can be easily torn at any region and the operculum popped-open at the P5 stage of development. Therefore, the GLD enzyme must in some unknown way act to modify the extracellular cuticular matrix resulting in a brittle cuticle allowing an easy exit for the adult upon completion of metamorphosis. In contrast to the larval epidermis, other ectodermal tissue including the imaginal discs, neural tissues, hindgut, and foregut do not express the *Gld* transcript in the third instar larva.

In the pharate adult (P7–8) *Gld* mRNA is abundantly expressed in several tissues derived from imaginal discs (Figure 7). Expression of *Gld* RNA is dispersed throughout the wing; whereas, it is restricted to joint regions of the legs. Similarly, specific regions of other tissues including the base of the antennae, the labium, thoracic and abdominal hypoderm, and the junction between the head and the thorax are found to express *Gld* mRNA. Internally, epidermal cells of the large tracheal trunks, the cibarium in the foregut, and the rectal papillae of the hindgut also express *Gld* mRNA. Inasmuch as all of these regions secrete a cuticle, it is possible that the GLD enzyme is involved in some modification of the cuticular matrix. However, any such modifications are nonessential since no obvious defects in these structures are observed in *Gld* mutants. It is important to note that the hybridization signal in the integument is specifically over the epidermal cells as opposed to nonspecific adherence to the adjacent cuticle. Results of parallel experiments using negative-control hybridization probes and experiments using RNAse treated sections support this interpretation.

In the pharate adult, *Gld* mRNA is abundantly expressed in the male ejaculatory duct and ejaculatory bulb and in the female oviduct and spermathecae. Because the switch in *Gld* expression from non-sex-limited to virtually male-limited does not occur until after eclosion (Figure 1, bottom), we did not anticipate the presence of *Gld* RNA in the developing reproductive tract of either sex. After eclosion, the expression of *Gld* mRNA and enzyme activity rises to very high levels in the male ejaculatory duct [Figures 1 and 8 (*5* and *6*)], and CAVENER *et al.* 1986). Low levels of *Gld* mRNA and enzyme activity are observed in females (approximately 20-fold less than males). Previously we

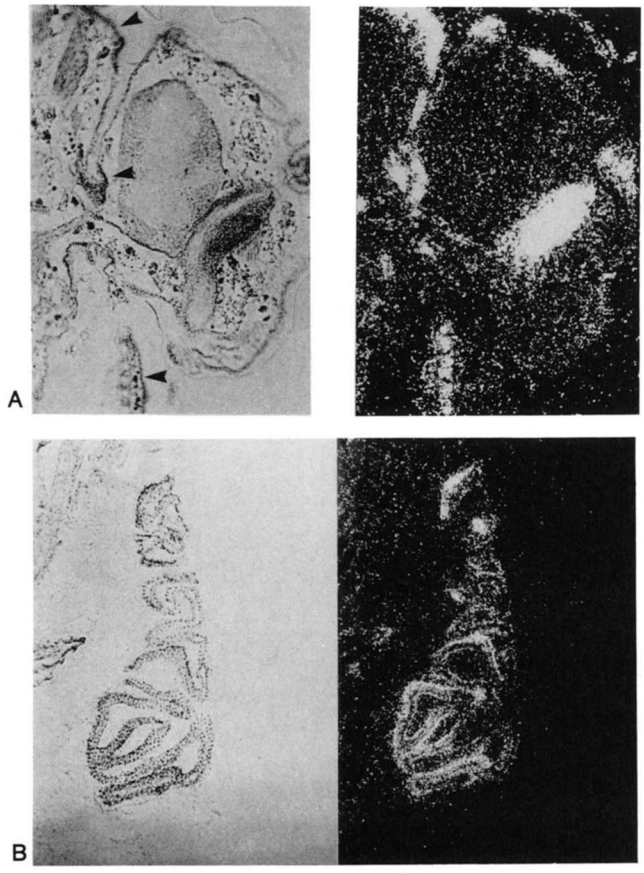

FIGURE 7.—*In situ* localization of *Gld* mRNA in P8–10 pharate adults. (A) Sagittal section of the head and anterior thorax showing hybridization signal over the dorsal thoracic hypoderm (left-upper arrow), neck (left-middle arrow), labium (left-lower arrow), antennal region (right-upper arrow), cibarium (right-lower arrow). (B) Section of a wing with signal dispersed throughout.

had assumed that the low level of *Gld* expression in adult females was a consequence of background expression of several or all tissues. However, we have recently discovered low but reproducible levels of GLD in the vaginal plate and spermathecal ducts in adult females.

DISCUSSION

Several aspects of the developmental regulation of *Gld* would not have been predicted from its genetically determined function. A thorough analysis of the temporal and spatial expression of *Gld* RNA revealed a surprisingly complex pattern, especially with respect to the variety of tissues which express *Gld*. During embryogenesis *Gld* RNA is observed in tissues associated with sensory organs (hypophysis and the antennal-maxillary complex). We speculate that the specific cells expressing *Gld* RNA within these tissues may be the epidermal cells known to secrete cuticular structures. However, we cannot exclude the possibility that *Gld* RNA may be expressed in the neuronal cells themselves. Later during the pharate adult stage, some of the developing sensory organs also express

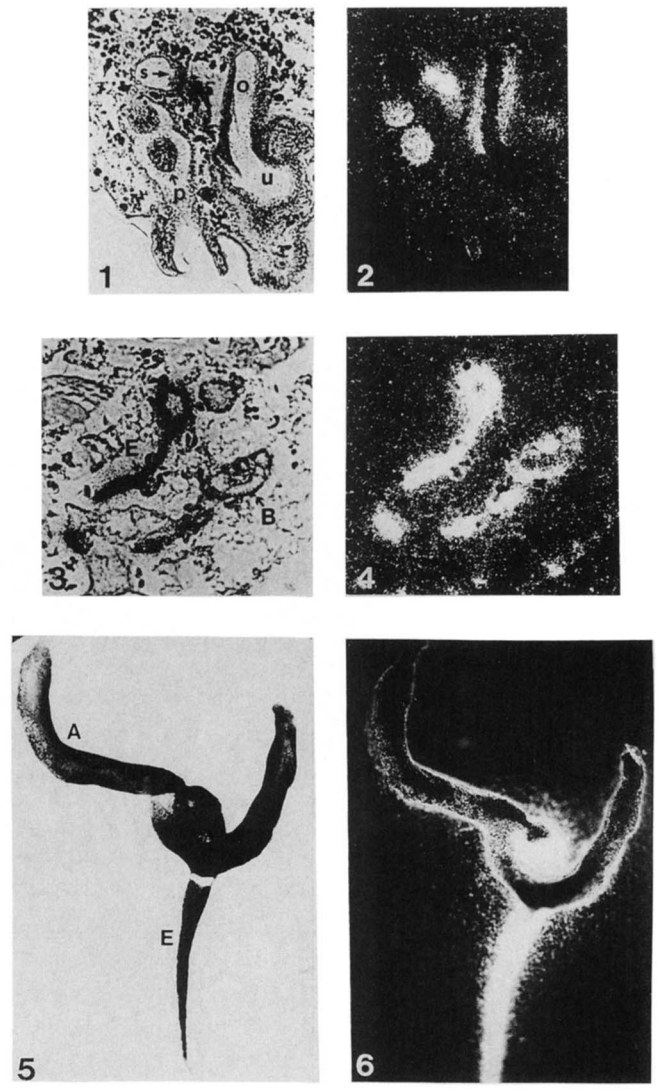

FIGURE 8.—*In situ* localization of *Gld* mRNA in the developing and mature reproductive tract. (*1* and *2*) Sagittal section of a female pharate adult showing hybridization signal over the rectal papillae (p), paired spermathecae (s), and oviduct (o). No signal is observed in the uterus (u). (*3* and *4*) Sagittal section of a male pharate adult showing hybridization signal over the ejaculatory duct (E) and the ejaculatory bulb (B). (*5* and *6*) Whole mount of a mature adult male reproductive tract showing intense hybridization signal over the entire anterior ejaculatory duct (E) but not the accessory glands (A).

Gld mRNA (labium, maxillary palps, antennae, and the cibarium). Presently, we have no evidence that *Gld* is expressed in the nervous system of either larvae or adults.

We propose that the temporal pattern of *Gld* expression during preadult development is largely, if not exclusively, controlled by ecdysteroids. The expression of *Gld* mRNA is temporally correlated with changes in ecdysteroid levels throughout preadult development. Furthermore, we have demonstrated that a genetic block in the accumulation of ecdysteroids represses *Gld* expression whereas *Gld* expression can be prematurely induced by feeding 20-hydroxy-ecdy-

sone (MURTHA and CAVENER 1989). Herein, we show that feeding hormone to third instar larvae leads to a large induction of *Gld* mRNA levels in the epidermal cells underlying the external cuticle and in the anterior spiracular gland cells. These tissues are also major sites of expression for the ecdysteroid inducible protein genes, Eip28/29 (A. ANDRES and P. CHERBAS, personal communication).

A few hours after pupariation of the third instar larva, GLD enzyme activity is detected in the presumptive molting fluid. We speculate that GLD is secreted from the epidermal cells and spiracular gland cells and then performs its only known essential function: modification of the puparium.

Examination of the catalytic function of GLD (conversion of glucose to γ-gluconolactone) has not led to an obvious biochemical model for GLD's apparent modification of the puparium. Currently the possibility that GLD may modify the carbohydrate moieties of glycoproteins within the cuticle is being investigated (D. COX-FOSTER, unpublished data). Preliminary experiments indicate an abnormal pattern of glycosylated proteins in the larval external cuticle and the puparium of *Gld* mutants. Because *Gld* mutants do not exhibit any other obvious defects, it is curious that GLD is expressed transiently at every stage of development and in several tissues. One possibility is that GLD may subtly modify a variety of cuticles throughout development; such modifications may be advantageous but nonessential. Conversely, the expression of GLD in these tissues may be solely dictated by the evolution of the underlying regulatory system and, therefore gratuitous relative to function (CAVENER 1987).

Perhaps the most surprising aspect of the developmental expression of *Gld* is the presence of mRNA in the developing reproductive tract of both males and females. Since mechanically rescued male and female *Gld* null mutants exhibit normal reproductive functions, the expression of *Gld* during the development of these organs is clearly not essential (CAVENER, OTTESON and KAUFMAN 1986). However, the origin of these four organs, the male ejaculatory duct and ejaculatory bulb and the female oviduct and spermathecae, is closely related; they are derived from the same region of the genital disc and, within each sex, derived from the same cell lineage (LAUGE 1980, 1982; DUBENDORFER and NOTHIGER 1982; EPPER and NOTHIGER 1982). We speculate that a regulatory circuit common to this cell lineage is responsible for the expression of *Gld*, and that upon sexual maturation their regulatory milieu becomes more distinctly organ-specific resulting in the loss of *Gld* expression in the oviduct and ejaculatory bulb at the adult stage.

Gld expression in the male ejaculatory duct was previously shown to be cell-autonomous with respect to sex by imaginal disc transplantation experiments (CAVENER 1985). Inasmuch as the sex-determination pathway of Drosophila is largely cell-autonomous (BAKER and RIDGE 1980), it seems that *Gld* expression would be influenced by the sex-determination pathway. However, unlike the expression of yolk proteins in the adult Drosophila fat body which requires continuous expression of the sex determining pathway in the female mode (BELOTE *et al.* 1985), *Gld* expression in the male ejaculatory duct and *mst 316* expression in the male accessory glands are not directly regulated by the sex determination pathway at the adult stage (CAVENER 1987; DIBENEDETTO *et al.* 1987). But recent experiments (Y. FENG and D. CAVENER, unpublished data) indicate that perturbations of the sex determination pathway during the third larval instar can affect subsequent organ-specific expression of *Gld* at the adult stage. We are currently investigating the relationship of the sex-determination pathway and the development of the relevant cell lineage to the expression of *Gld* in the reproductive tract.

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