

## Steroid regulated programmed cell death during *Drosophila* metamorphosis

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### SUMMARY

During insect metamorphosis, pulses of the steroid hormone 20-hydroxyecdysone (ecdysone) direct the destruction of obsolete larval tissues and their replacement by tissues and structures that form the adult fly. We show here that larval midgut and salivary gland histolysis are stage-specific steroid-triggered programmed cell death responses. Dying larval midgut and salivary gland cell nuclei become permeable to the vital dye acridine orange and their DNA undergoes fragmentation, indicative of apoptosis. Furthermore, the histolysis of these tissues can be inhibited by ectopic expression of the baculovirus anti-apoptotic protein p35, implicating a role for caspases in the death response. Coordinate stage-specific induction of the *Drosophila* death genes *reaper* (*rpr*) and *head involution*

*defective* (*hid*) immediately precedes the destruction of the larval midgut and salivary gland. In addition, the *diap2* anti-cell death gene is repressed in larval salivary glands as *rpr* and *hid* are induced, suggesting that the death of this tissue is under both positive and negative regulation. Finally, *diap2* is repressed by ecdysone in cultured salivary glands under the same conditions that induce *rpr* expression and trigger programmed cell death. These studies indicate that ecdysone directs the death of larval tissues via the precise stage- and tissue-specific regulation of key death effector genes.

Key words: steroid hormones, apoptosis, p35, metamorphosis, ecdysone, *Drosophila*, *reaper*, *head involution defect*, *hid*, *rpr*

### INTRODUCTION

The development of higher organisms entails growth and differentiation along with the orderly and directed destruction of obsolete cells. Programmed cell death usually proceeds through a stereotypical series of distinct morphological stages that include cellular condensation, DNA fragmentation and the formation of apoptotic bodies (Kerr et al., 1972; Wyllie, 1980). Because apoptosis plays such a critical role in both normal and abnormal development, extensive studies have focused on its regulation. These studies have led to the identification of genes that function at different levels in the apoptotic pathway, to either induce or prevent cell death. Complementary studies in *C. elegans*, *D. melanogaster* and mammalian cell culture have revealed that many of these critical regulators have been conserved through evolution (Steller, 1995; Jacobson et al., 1997). It thus seems likely that genetic pathways defined in simple model organisms will provide a foundation for understanding the regulation of programmed cell death in more complex organisms.

One of the first studies of programmed cell death characterized insect intersegmental muscle degeneration (Lockshin and Williams, 1964, 1965) in response to the ecdysone pulse that signals the onset of metamorphosis. The motoneurons that innervate the larval proleg muscles of the tobacco hornworm, *Manduca sexta*, also degenerate at this time (Weeks and Truman, 1985) as do the labial glands, a homolog of the

*Drosophila* salivary glands (Lockshin and Zakeri, 1994). A second round of programmed cell death takes place at the end of metamorphosis in response to a decrease in ecdysone titer. These responses include the death of abdominal interneurons and motoneurons as well as the destruction of the abdominal intersegmental muscle (Finlayson, 1956; Schwartz and Truman, 1982; Truman et al., 1992).

Studies in *Drosophila* have revealed that apoptosis can also be detected during embryogenesis, affecting groups of cells that lie in different regions of the embryo (Abrams et al., 1993). These responses are essential for viability, since mutations that reduce or eliminate cell death result in lethality during embryogenesis with defects in head involution (Abbott and Lengyel, 1991; White et al., 1994). Programmed cell death is also an integral aspect of *Drosophila* eye differentiation, which depends on the ordered elimination of extra cells between the ommatidia (Cagan and Ready, 1989; Wolff and Ready, 1991). In a similar manner, a set of approximately 300 neurons in the central nervous system undergo programmed cell death following adult eclosion (Truman et al., 1992, 1994). These cells selectively express the EcR-A isoform of the ecdysone receptor and their death is dependent on a decrease in ecdysone titer, implicating the hormone as a primary signal for this response (Robinow et al., 1993; Talbot et al., 1993).

A genetic framework for understanding the regulation of programmed cell death in insects came from molecular characterization of an ~300 kb region in the *Drosophila* genome

that is required for cell death during embryonic development (White et al., 1994). Three death genes have been identified within this interval: *rpr*, *hid* and *grim* (White et al., 1994; Grether et al., 1995; Chen et al., 1996). The induction of each of these genes foreshadows apoptosis during embryogenesis, although *hid* is also expressed in some cells that survive. Mutations in *hid* result in a reduction in programmed cell death that leads to embryonic lethality (Grether et al., 1995), and ectopic expression of any of these genes is sufficient to trigger apoptosis (Grether et al., 1995; Hay et al., 1995; Chen et al., 1996; White et al., 1996). The *rpr* gene encodes a polypeptide that has limited sequence identity with the family of 'death domain' proteins defined by the mammalian tumor necrosis factor receptor (TNFR) family (Cleveland and Ihle, 1995; Golstein et al., 1995). In addition, *rpr*, *hid* and *grim* activate a death program that is mediated by caspases, similar to the pathway triggered by TNFR 1 and Fas in vertebrate cells (Pronk et al., 1996; White et al., 1996). This response can be blocked by ectopic expression of the baculovirus p35 gene which functions to inhibit caspase activity (Bump et al., 1995; Xue and Horvitz, 1995). Taken together, these observations argue that at least some aspects of the regulation of apoptosis have been conserved from *Drosophila* to vertebrates. Finally, several genes related to baculovirus IAPs (Inhibitors of Apoptosis) have been identified in the *Drosophila* genome (Hay et al., 1995; Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996). At least some members of the IAP family block cell death in a manner similar to that of p35 (Crook et al., 1993; Birnbaum et al., 1994), by directly inhibiting caspase activity (Deveraux et al., 1997). The ability of the *Drosophila* IAP proteins to block cell death induced by *rpr* or *hid* suggests that they may normally function as negative regulators of programmed cell death during development (Hay et al., 1995).

Steroid hormones are recognized as one of many elicitors of programmed cell death in higher organisms (Evans-Storms and Cidlowski, 1995; Steller, 1995). The death of lymphoid cells in response to glucocorticoids has provided the primary model for these studies, via a pathway that is mediated, at least in part, by the glucocorticoid receptor (Wyllie, 1980; Ucker, 1987; Nazareth et al., 1991; Dieken and Miesfeld, 1992; Helmsberg et al., 1995). The steps by which the hormone-receptor complex initiates cell death, however, remain unknown (Evans-Storms and Cidlowski, 1995).

We have been studying the regulation of programmed cell death during *Drosophila* metamorphosis as a model system for understanding how steroid hormones trigger this response during development. Metamorphosis in *Drosophila* is regulated by changes in the titer of the steroid hormone ecdysone. A pulse of ecdysone at the end of larval development triggers puparium formation and the onset of prepupal development, followed 10 hours later by another ecdysone pulse that signals pupation, defining the prepupal-pupal transition (Riddiford, 1993). The larval tissues undergo histolysis during prepupal and early pupal development, as adult structures grow and differentiate from clusters of imaginal progenitor cells (Robertson, 1936; Bodenstein, 1965). The destruction of the larval tissues proceeds in a precise stage- and tissue-specific manner following each ecdysone pulse. For example, the anterior larval muscles and larval midgut degenerate during the first half of prepupal development, while some abdominal

muscles and the larval salivary glands undergo histolysis shortly after pupation.

In this study, we focus on two larval tissues that undergo histolysis at different times and in different ways – the larval midgut and salivary glands (Robertson, 1936; Bodenstein, 1965). The larval midgut contains polyploid larval epithelial cells that lie on an external basement membrane. Adjacent to the basement membrane are small islands of diploid imaginal cells that will form the adult midgut. These progenitor cells proliferate during early prepupal development forming a new cell layer. The result is an adult midgut that surrounds a condensed mass of larval cells, referred to as the yellow body, which is discharged as the meconium soon after eclosion. The salivary gland, in contrast, undergoes a much more abrupt transition, degenerating rapidly by ~15 hours after puparium formation (Robertson, 1936). A ring of diploid imaginal cells lies at the anterior end of the salivary gland and serves as a source of cells that form the adult gland during pupal development.

*Drosophila* larval salivary gland histolysis provides an ideal opportunity to understand the steroid regulation of programmed cell death. Salivary gland histolysis is dependent on ecdysone (Aizenzon and Zhimulev, 1975) and is nearly synchronous among the cells that constitute this tissue (Bodenstein, 1943). In addition, an ecdysone-triggered genetic regulatory hierarchy has been characterized that immediately precedes the death of this tissue (Richards, 1976; Woodard et al., 1994; Baehrecke and Thummel, 1995; White et al., 1997). This two-step regulatory hierarchy is composed of early and late genes, defined by the puffing patterns of the salivary gland polytene chromosomes (Ashburner et al., 1974). The early genes are induced directly by the ecdysone-receptor complex and encode a variety of proteins, including transcription factors that induce late gene expression (Russell and Ashburner, 1996; Thummel, 1996). The late genes, in turn, appear to play a more direct role in salivary gland development and function. Similar regulatory hierarchies are triggered by ecdysone in other target tissues, providing an opportunity to understand how the hormone coordinates different developmental pathways during metamorphosis.

We show here that the destruction of the larval midgut and salivary glands is accompanied by nuclear staining with acridine orange and DNA fragmentation, indicative of the hallmark features of apoptosis. Larval midgut and salivary gland histolysis can be inhibited by ectopic expression of p35, suggesting that larval cell death depends on the activation of caspases. Furthermore, expression of the death inducers *rpr* and *hid* and repression of the death inhibitor *diap2* correlates with the onset of histolysis in the larval salivary gland, suggesting that programmed cell death may be coordinated by both inductive and repressive mechanisms. Based on these observations, we propose a model for how ecdysone-induced transcription factors may direct the death response through the stage- and tissue-specific regulation of these death effector genes.

## MATERIALS AND METHODS

### Developmental staging

Late third instar larvae were staged by growth on food containing

0.1% bromophenol blue, as described (Andres and Thummel, 1994). Blue gut mid-third instar larvae are referred to as -18 hours relative to puparium formation, partially clear gut late third instar larvae are referred to as -8 hour, and clear gut late third instar larvae are referred to as -4 hour. Prepupae and pupae are staged relative to puparium formation (0 hour).

### Detection of cell death

Dissected midguts or salivary glands were incubated for 5 minutes in 5 µg/ml acridine orange in phosphate-buffered saline (PBS) (Abrams et al., 1993). The organs were then placed in fresh PBS and analyzed immediately for nuclear staining on a BioRad MRC-1024 confocal laser scanning microscope. Images were generated by overlaying a z-series of 20-30 4 µm sections, each of which was Kalman averaged.

TUNEL assays were performed using the Boehringer Mannheim in situ cell death detection kit AP, essentially as described by the manufacturer. Cryosections of staged prepupae or pupae were fixed in 4% paraformaldehyde in PBS for 20 minutes and digested with 25 µg/ml proteinase K in PBS for 15 minutes at 25°C to increase their permeability. After re-fixation, the sections were incubated in 50 µl TUNEL reaction mixture (containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-conjugated dUTP) for 1 hour at 37°C to label the ends of DNA fragments. The labeled ends were detected by incubating first in 50 µl alkaline phosphatase (AP)-conjugated anti-fluorescein antibody for 30 minutes at 37°C and next in 100 µl vector red AP substrate solution (Vector Laboratories) for 40 minutes at 25°C. After counterstaining with 0.3% methyl green, 0.1 M NaOAc, pH 4.0, the sections were mounted in Permount and examined by light microscopy.

### Ectopic p35 expression

The Glass/GMR system was used to study the effects of ectopic p35 expression on larval midgut histolysis (Hay et al., 1994). Homozygous *hs-glass* flies were crossed with either homozygous *GMR-p35* flies, or *w<sup>1118</sup>* flies as a negative control. Late third instar larvae from these crosses were heat shocked at 37°C for 30 minutes and allowed to recover at 25°C for 4 hours. Newly formed prepupae were then selected from this population, allowed to develop for 4 hours at 25°C and dissected to examine the morphology of their larval midguts. Northern blot analysis revealed that p35 mRNA began to increase 2 hours after heat shock and reached peak levels by 3-4 hours (data not shown).

The Gal4/UAS system was used to study the effects of ectopic p35 expression on larval salivary gland histolysis (Brand and Perrimon, 1993; Gustafson and Boulianne, 1996). D59 flies, which express GAL4 at high levels in the salivary glands, were crossed with either homozygous *UAS-p35* flies or *w<sup>1118</sup>* flies as a negative control. White prepupae from these crosses were collected and allowed to develop at 25 °C for 14, 15, 16, 18, 20 or 22 hours. These animals were then dissected to examine the morphology of their larval salivary glands.

### Ecdysone injections

Larvae were injected with ecdysone essentially as described by Mitchell (1978). 100 blue gut mid-third instar larvae were collected, washed with PBS, etherized for 4 minutes and mounted on double-sided tape along the edge of six slides. Half of the animals were injected with 0.1-0.2 µl of 1 mM 20-hydroxyecdysone (Sigma), 5% ethanol, 0.05% green food dye (Carlin Foods) in Graces insect medium (BRL/GIBCO), while the other half received the same volume of the same solution without 20-hydroxyecdysone. The larvae were released from the tape by rinsing with PBS and transferred to yeast paste. Whereas 87% of the control larvae pupariated >17 hours after injection, 82% of the ecdysone-injected larvae pupariated 6-8 hours after injection. Extensive cell death can be detected in the midguts of animals 8-10 hours after ecdysone injection (1-2 hours after pupariation) as assayed by acridine orange staining.

### Northern blot hybridization

RNA isolated from staged whole animals, dissected midguts, dissected salivary glands or cultured salivary glands were prepared as described previously (Andres and Thummel, 1994). Equal amounts of total RNA were electrophoresed on formaldehyde-agarose gels, transferred to a nylon membrane (Genescreen, Dupont) and hybridized to detect *rpr*, *hid* or *diap2* transcription. Probes were synthesized from *rpr* and *hid* cDNAs provided by H. Steller (White et al., 1994; Grether et al., 1995), a *grim* cDNA provided by J. Abrams (Chen et al., 1996), or a 561 bp *ClaI* fragment from a *diap2* cDNA provided by B. Hay (Hay et al., 1995). The blots were hybridized and washed as described (Baehrecke and Thummel, 1995).

## RESULTS

### Histolysis of the larval midgut and salivary glands

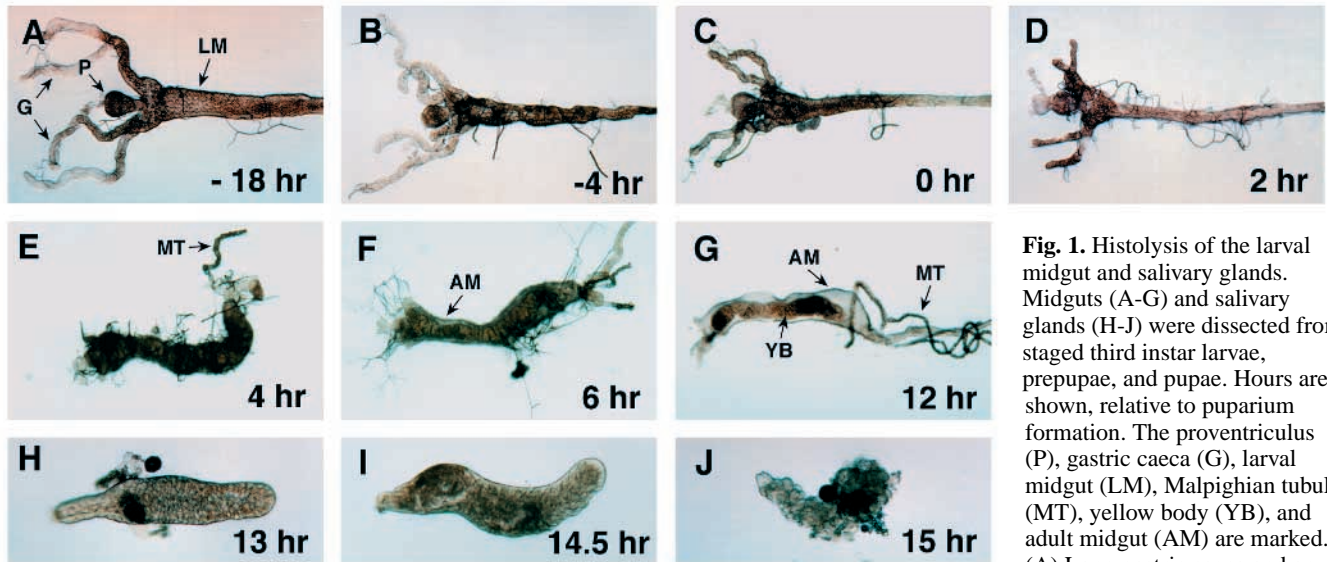
We examined the morphology of the larval midgut and salivary glands during the onset of metamorphosis in order to provide a foundation for our studies of larval tissue histolysis (Fig. 1). The midguts of feeding mid-third instar larvae are fully expanded and the outlines of the polyploid larval cells can be easily distinguished (Fig. 1A). The esophagus extends through the bulb-shaped proventriculus (P in Fig. 1A) into the midgut (LM in Fig. 1A). Four diverticuli extend off of the anterior end of the midgut, forming the gastric caeca (G in Fig. 1A). The midgut condenses slightly during late larval development, perhaps due to the cessation of feeding. This is evident in late third instar larvae that are approximately 4 hours prior to puparium formation (Fig. 1B). The first distinct morphological change can be seen at puparium formation, when the gastric caeca are noticeably shorter (Fig. 1C). This is followed by a dramatic change in gut morphology between 2 and 4 hours after puparium formation – the midgut contracts to a fraction of its former length, the gastric caeca disappear and the proventriculus is significantly reduced in size (Fig. 1E). A layer of adult midgut cells can be distinguished by 6 hours after puparium formation, surrounding the condensing larval midgut (AM in Fig. 1F). The adult midgut elongates during the next 6 hours and the larval midgut continues to condense to form the yellow body (YB in Fig. 1G).

In contrast, the larval salivary glands appear relatively unchanged until after the ecdysone pulse that occurs ~10 hours after puparium formation. The cells initially detach from the basement membrane that surrounds the gland and are noticeably degraded by 14.5 hours after puparium formation (Fig. 1H,I). The gland then degrades rapidly over the next 30 minutes (Fig. 1J), until it is no longer detectable.

### Larval midgut and salivary gland histolysis is accompanied by DNA fragmentation and nuclear permeability to acridine orange

Although it seems logical that *Drosophila* larval tissue histolysis represents a steroid-triggered programmed cell death response, no studies have been performed to address this issue. As an initial test of this hypothesis, we assayed whether larval midgut and salivary gland histolysis is accompanied by nuclear permeability to acridine orange, a vital dye that provides a rapid and accurate indicator of apoptosis in insect tissues (Spreij, 1971; Abrams et al., 1993). Variable low levels of nuclear staining could be detected in the gastric caeca and





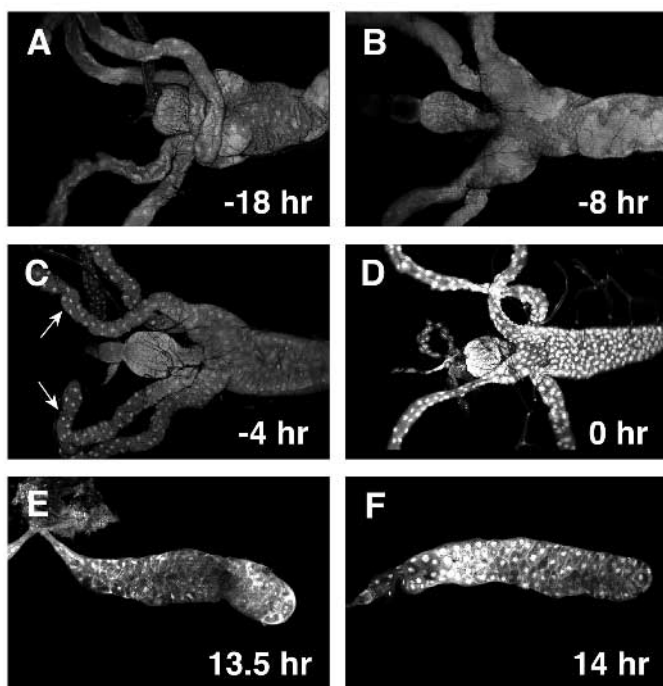
**Fig. 1.** Histolysis of the larval midgut and salivary glands. Midguts (A-G) and salivary glands (H-J) were dissected from staged third instar larvae, prepupae, and pupae. Hours are shown, relative to puparium formation. The proventriculus (P), gastric caeca (G), larval midgut (LM), Malpighian tubules (MT), yellow body (YB), and adult midgut (AM) are marked. (A) Long gastric caeca and an extended midgut are present in

feeding mid-third instar larvae. (B) The gastric caeca remain elongated in late third instar larvae as the midgut begins to condense. (C,D) The gastric caeca shorten in newly formed prepupae and the midgut grows thinner. (E) The proventriculus is significantly reduced in size by 4 hours after puparium formation, the gastric caeca are no longer detectable, and the midgut is highly contracted. (F) The midgut elongates as the adult cells proliferate. (G) The condensed larval midgut can be clearly seen inside the developing adult midgut. (H) Normal salivary glands are present in 13 hour pupae. (I) Salivary glands show signs of significant degeneration by 14.5 hours after puparium formation. (J) Histolysis is virtually complete 30 minutes later.

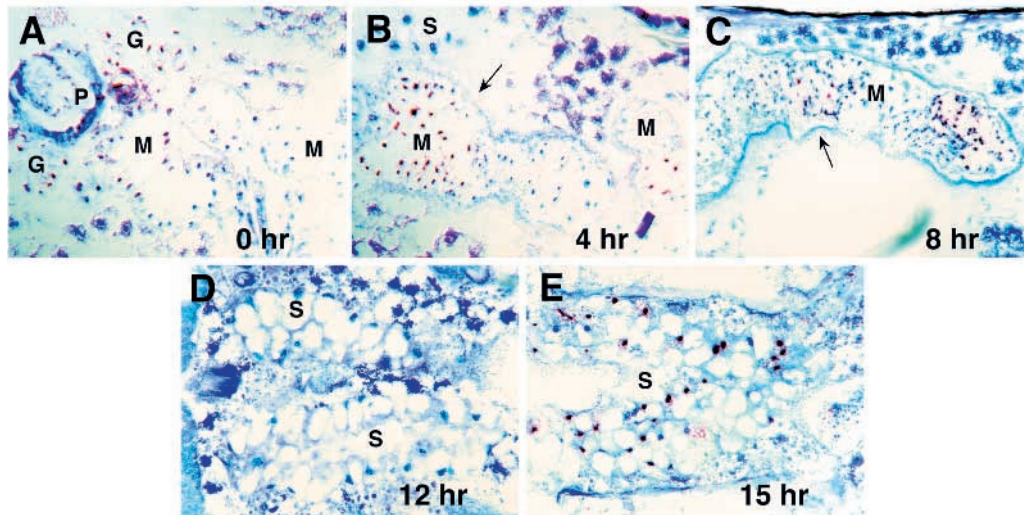
midguts of mid- and late third instar larvae (Fig. 2A,B). In contrast, a reproducible nuclear stain was observed in the gastric caeca of late third instar larvae (Fig. 2C), immediately preceding the shortening of these structures that occurs at the end of larval development (Fig. 1B,C). An intense nuclear stain was present in the gastric caeca and midguts of newly formed prepupae, foreshadowing the destruction of these tissues (Fig. 2D).

Larval salivary glands dissected from prepupae revealed no nuclear staining by acridine orange (data not shown). By 13.5 hours after puparium formation, variable nuclear staining could be detected (Fig. 2E), whereas a uniform nuclear stain was observed in salivary glands isolated from 14 hour pupae (Fig. 2F).

Sections from staged prepupae and early pupae were stained using the TUNEL assay in order to determine whether larval midgut and salivary gland cell death is accompanied by DNA fragmentation (Gavrieli et al., 1992). We used a red alkaline phosphatase substrate and methyl green as a counterstain in order to more readily detect dying cells (Fig. 3). A strong TUNEL stain could be observed in the gastric caeca and anterior midgut of newly formed prepupae (Fig. 3A), as these structures are shortening and condensing (Fig. 1C). In contrast, no TUNEL staining was evident in more posterior regions of the larval midgut (right, Fig. 3A) until 4-8 hours after puparium formation (Fig. 3B,C). The adult midgut cells could be clearly distinguished by these stages, surrounding the condensing

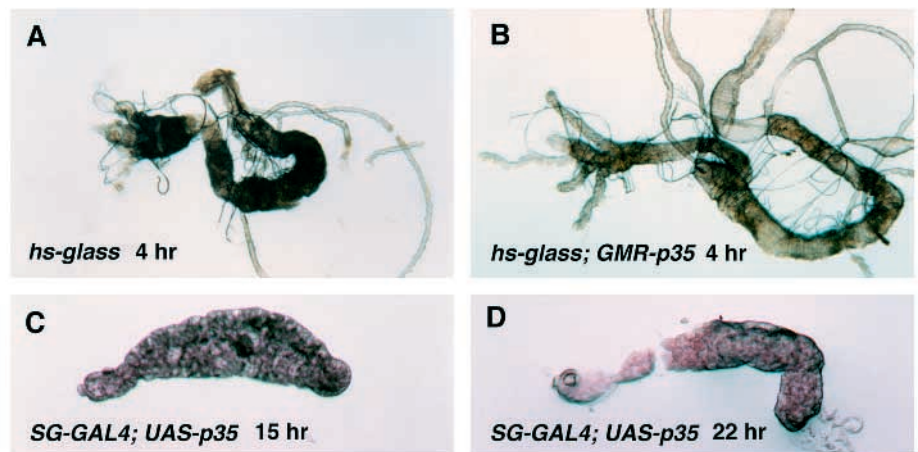


**Fig. 2.** Nuclear acridine orange staining precedes larval midgut and salivary gland histolysis. Midguts (A-D) and salivary glands (E,F) dissected from staged third instar larvae, prepupae, and pupae were stained with acridine orange to visualize the nuclear permeability associated with programmed cell death. (A,B) Midguts from mid (-18 hour) and late (-8 hour) third instar larvae displayed low levels of staining in some nuclei. (C) Late third instar larvae (approximately 4 hours prior to pupariation) displayed a reproducible low level of nuclear stain in the gastric caeca (arrows). (D) Newly formed prepupal midguts revealed intense nuclear acridine orange staining. (E) Occasional nuclear staining was observed in salivary glands isolated from 13.5 hour prepupae. (F) In contrast, a uniform nuclear stain could be detected in salivary glands isolated from 14 hour pupae.



**Fig. 3.** Programmed cell death in the larval midgut and salivary glands is associated with DNA fragmentation. Cryosections of staged prepupae were stained using the TUNEL assay to detect DNA fragmentation associated with programmed cell death. A red nuclear stain indicates the presence of fragmented DNA while living cells stain blue or purple. Times are shown relative to puparium formation. (A) Staining is evident in the gastric caeca and anterior midgut of newly formed prepupae, but not in more posterior midgut regions (right). (B,C) Sections from 4 and 8 hour prepupae reveal the adult gut (arrow) developing around the condensing larval midgut cells, many of which are stained. (D) Salivary gland nuclei are not stained by the TUNEL assay in 12 hour prepupae. (E) By 15 hours after pupariation, all salivary gland nuclei are intensely stained. Salivary glands are labeled (S), as well as regions of the gut: proventriculus (P), gastric caeca (G), and midgut (M).

**Fig. 4.** Programmed cell death is inhibited by expression of the baculovirus p35 protein. Late third instar larvae carrying either the *hs-glass* P element construct or *hs-glass* and *GMR-p35* were heat shocked 4 hours before puparium formation and allowed to recover for 8 hours. Midguts were then dissected from these 4 hour prepupae and examined. Six animals were examined and all gave similar results. (A) Midguts expressing *hs-glass* were significantly condensed and contained very short gastric caeca, resembling wild-type midguts (Fig. 1E). (B) Midguts expressing p35 were significantly longer and had longer gastric caeca, resembling midguts normally seen in earlier prepupae (Fig. 1C,D). (C) Salivary glands that expressed p35 under the control of the GAL4/UAS system were not histolyzed in 15 hour prepupae, when the salivary glands are usually highly degraded (Fig. 1J). (D) Salivary glands that express p35 could still be detected by 22 hours after puparium formation.



TUNEL-positive larval cells (arrows, Fig. 3B,C). As expected, the salivary glands show no TUNEL staining throughout prepupal development (Fig. 3D), but a strong and uniform TUNEL stain can be detected by 15 hours after puparium formation, as the gland undergoes the final stages of histolysis.

#### Expression of baculovirus p35 inhibits larval midgut and salivary gland cell death

Expression of the baculovirus p35 protein can block apoptosis in a wide variety of organisms, including mammals, nematodes and flies (Rabizadeh et al., 1993; Hay et al., 1994; Sugimoto et al., 1994). This protein functions by binding to caspases and

preventing their activation by proteolysis (Bump et al., 1995; Xue and Horvitz, 1995). In order to determine if p35 can also inhibit the death of larval midgut and salivary gland cells, we expressed p35 in these tissues and then assayed the effects on histolysis.

The *glass/GMR* system was used to induce p35 expression in the midguts of early prepupae (Hay et al., 1994). Late third instar larvae carrying either the *hs-glass* construct, or both *hs-glass* and *GMR-p35*, were heat shocked and allowed to recover for 4 hours at room temperature. Newly formed prepupae were selected from these populations of animals and allowed to develop for an additional 4 hours, after which the midguts were dissected and examined. Midguts from 4 hour prepupae

expressing only *hs-glass* closely resemble those seen in wild-type 4 hour prepupae (compare Figs 4A and 1E). In contrast, midguts from 4 hour prepupae that express p35 appear to be blocked in their histolysis (Fig. 4B). These midguts are elongated and contain detectable gastric caeca, resembling the midguts of wild-type 0-2 hour prepupae (Fig. 1C,D).

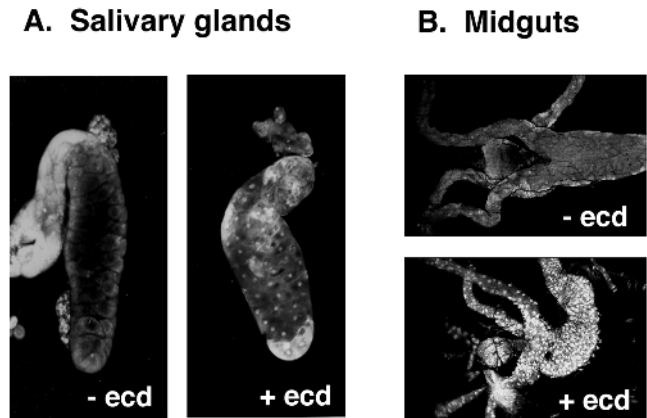
The GAL4 expression system was used to selectively express p35 in the larval salivary glands (Brand and Perrimon, 1993; Gustafson and Boulianne, 1996). Flies carrying a *UAS-p35* P element construct were crossed with an enhancer trap line that drives GAL4 expression in the larval salivary glands. Staged early pupae were selected from these animals and their salivary glands were dissected and examined. Salivary glands could be easily identified in 15 hour pupae, a time when they are almost impossible to dissect in wild-type animals (Fig. 4C). Furthermore, salivary glands could still be identified 7 hours later, long after histolysis is normally complete (Fig. 4D). The rescued salivary gland cells were, however, abnormal in morphology and appeared to be partially degraded. Consistent with this possibility, the nuclei of these cells could be stained with acridine orange (data not shown). It is possible that higher levels of p35 expression are required to completely block salivary gland cell death.

#### Ecdysone triggers cell death in larval salivary glands and midguts

Larval salivary glands can be maintained for many hours in organ culture, providing an ideal opportunity to study the hormonal requirements for a variety of responses to ecdysone, including glue secretion, polytene chromosome puffing and specific gene regulation (Ashburner, 1972; Boyd and Ashburner, 1977). In order to determine if larval salivary gland histolysis is an ecdysone-triggered response, we cultured mid-prepupal salivary glands for 5-7 hours in the presence or absence of ecdysone, and then stained them with acridine orange. Most glands cultured in the absence of ecdysone for 7 hours showed no detectable nuclear stain (72%,  $n=25$  glands) or a light nuclear stain (20%). In contrast, the majority of glands cultured in the presence of ecdysone for 7 hours showed a strong nuclear acridine orange stain (87%,  $n=31$  glands). This distinction can be seen as early as 5 hours after ecdysone addition (Fig. 5A). These observations indicate that salivary gland cell death is an ecdysone-triggered response.

A similar attempt to detect cell death in cultured larval midguts was unsuccessful, most likely due to the lack of appropriate culture conditions for this tissue. We tried several culture conditions similar to those used for salivary glands (Ashburner, 1972; Woodard et al., 1994). Within several hours of culture, however, the midguts contract significantly and the larval cells change their morphology (data not shown). Some midgut cells also display a nuclear acridine orange stain after a few hours in culture, in either the presence or absence of ecdysone. Unlike the larval salivary gland, larval midguts are a complex tissue composed of multiple cell types including epithelial cells, muscle and trachea. It seems likely that maintaining these cell types in culture will require the development of new organ culture conditions.

We thus assayed the effects of injecting ecdysone on larval gut histolysis. Crawling mid-third instar larvae were anesthetized with ether and injected with either 20-hydroxyecdysone or the same solution missing the hormone.



**Fig. 5.** Ecdysone triggers programmed cell death in larval salivary glands and midguts. (A) Salivary glands dissected from 8 hour prepupae were cultured in the absence (-ecd) or presence (+ecd) of  $5 \times 10^{-6}$  M 20-hydroxyecdysone for 5 hours and tested for viability by incubation with acridine orange. Only salivary glands cultured with ecdysone undergo cell death as indicated by nuclear staining. (B) Mid-third instar larvae were injected with either 20-hydroxyecdysone (+ecd) or the same solution missing the hormone (-ecd). Midguts were dissected after 8-10 hours and tested for viability by incubation with acridine orange. Only midguts from ecdysone-injected larvae enter cell death, as indicated by nuclear staining.

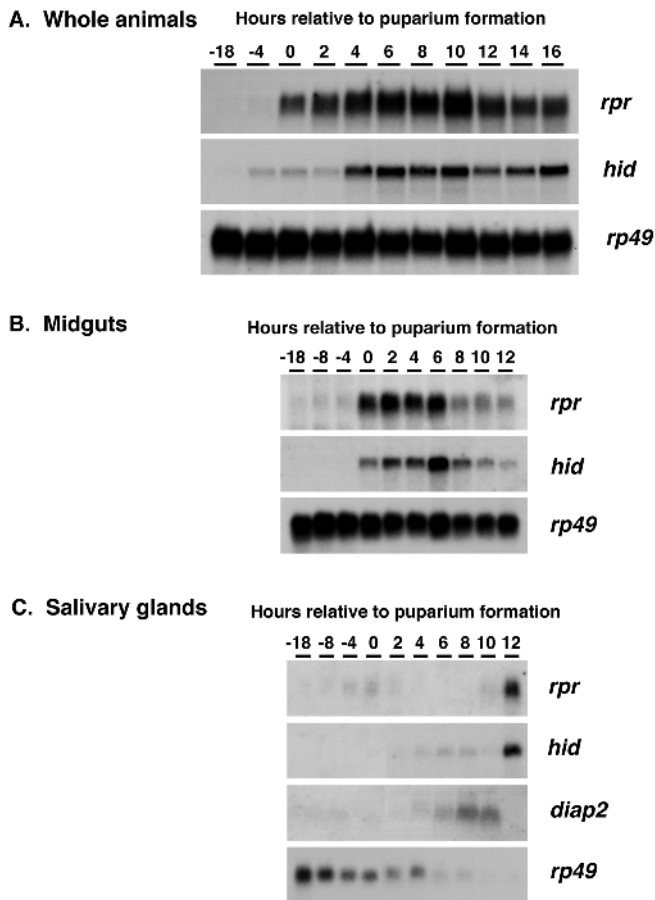
Ecdysone-injected larvae pupariated within 6-8 hours whereas control animals remained as third instar larvae for up to 18 hours. Midguts were dissected from either control or ecdysone-injected animals 8-10 hours after injection and stained with acridine orange. Only midguts isolated from ecdysone-injected animals showed a uniform nuclear acridine orange stain, indicative of the onset of programmed cell death (Fig. 5B).

#### *rpr* and *hid* induction immediately precedes larval midgut and salivary gland cell death

We wanted to determine whether we could link the destruction of larval tissues to the expression of known *Drosophila* cell death regulators. Total RNA was isolated from either intact animals, midguts or salivary glands of staged late third instar larvae and prepupae. Equal amounts of RNA were fractionated by formaldehyde agarose gel electrophoresis and analyzed by northern blot hybridization using probes specific for the *rpr*, *hid* or *grim* death genes (Fig. 6). No *grim* transcription could be detected at these stages, suggesting that this gene does not function during metamorphosis (data not shown). In contrast, both *rpr* and *hid* mRNA are expressed throughout prepupal and early pupal development, correlating with the timing of larval tissue histolysis (Fig. 6A). *hid* mRNA can first be detected in late third instar larvae, several hours before the induction of *rpr* at puparium formation. The levels of *hid* then increase in 4 hour prepupae, perhaps reflecting the destruction of the anterior larval muscles (Bodenstein, 1965).

By examining RNA isolated from either staged larval midguts or salivary glands, it is clear that *rpr* and *hid* are induced at different times in these tissues, immediately preceding the onset of programmed cell death. Both *rpr* and *hid* are coordinately induced in the larval midgut at puparium formation and are down-regulated between 6 and 8 hours after

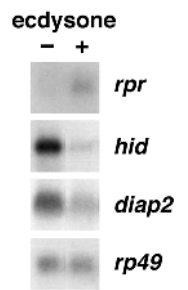




**Fig. 6.** The expression of genes that regulate programmed cell death correlates with the onset of histolysis in the larval midguts and salivary glands. RNA was isolated from either whole staged animals (A), dissected midguts (B) or salivary glands (C), and analyzed by northern blot hybridization to detect the patterns of *rpr*, *hid* and *diap2* transcription. (A) *rpr* and *hid* are induced at puparium formation and expressed throughout prepupal development, consistent with the timing of larval tissue histolysis. The *hid* gene is induced several hours before *rpr*, in late third instar larvae. (B) *rpr* and *hid* are induced in the midguts of newly formed prepupae and expressed during early prepupal development, correlating with the onset of programmed cell death. (C) *rpr* and *hid* are induced in salivary glands from 12 hour pupae, immediately preceding cell death. In contrast, *diap2* mRNA is induced in mid-prepupae and repressed as *rpr* and *hid* are induced. Hybridization to detect *rp49* mRNA was used as a control for loading and transfer. Since *rp49* is normally down-regulated in prepupal salivary glands, ethidium bromide staining prior to transfer was used to determine that equal amounts of RNA were present in these lanes (data not shown). The whole animal and salivary gland northern blots have been used previously to examine the transcription of other ecdysone-regulated genes (Karim and Thummel, 1992; Woodard et al., 1994; Baehrecke and Thummel, 1995).

puparium formation (Fig. 6B), paralleling the dramatic changes in midgut morphology that occur at these times. We assume that the light acridine orange stain seen in the gastric caeca of late third instar larval midguts (Fig. 2C) is not reflected in the patterns of *rpr* and *hid* transcription because these cells comprise such a small proportion of the midgut tissue dissected for RNA extraction. Both *rpr* and *hid* are also

**Fig. 7.** Ecdysone regulation of *rpr*, *hid* and *diap2* transcription in cultured salivary glands. Salivary glands dissected from 8 hour prepupae were cultured for 5 hours in the absence (-) or presence (+) of  $5 \times 10^{-6}$  M 20-hydroxyecdysone, after which RNA was extracted and analyzed by northern blot hybridization. *rpr* transcription is induced by the hormone, while *hid* and *diap2* are repressed. Hybridization to detect *rp49* mRNA was used as a control for loading and transfer.



coordinately induced in salivary glands dissected from 12 hour prepupae (Fig. 6C), 2 hours after the prepupal ecdysone pulse and 3 hours before salivary gland histolysis.

We also examined the temporal profiles of *diap1* and *diap2* transcription during the onset of metamorphosis (Hay et al., 1995). Both genes are expressed at low levels in late larvae and prepupae, in both whole animals and staged midguts (data not shown). In contrast, *diap1* transcription is undetectable in salivary glands while *diap2* revealed a distinct pattern of expression (Fig. 6C). This anti-apoptosis gene is induced in mid-prepupal salivary glands and abruptly repressed in early pupae, as *rpr* and *hid* are induced.

#### Ecdysone directs *diap2* repression and *rpr* induction in cultured larval salivary glands

An ecdysone-triggered regulatory hierarchy has been defined in the salivary glands of late prepupae (Richards, 1976; Woodard et al., 1994; Baehrecke and Thummel, 1995). In an initial effort to integrate death gene expression into this regulatory hierarchy, we examined the temporal patterns of *rpr*, *hid* and *diap2* transcription in cultured salivary glands. Glands were dissected from 8 hour prepupae and cultured for 5 hours in either the absence or presence of ecdysone. RNA was then isolated from these glands and analyzed by northern blot hybridization (Fig. 7). *rpr* transcription is induced by ecdysone in these cultured glands and *diap2* is repressed, paralleling the responses seen in salivary glands isolated from staged animals (Fig. 6C). In contrast, *hid* is expressed in prepupal salivary glands cultured in the absence of ecdysone and appears to be repressed by ecdysone in this assay (Fig. 7). Similar discrepancies between ecdysone-regulated gene expression in vitro and in vivo have been reported (see Ashburner, 1972; Hurban and Thummel, 1993, for discussions of this issue). Northern blot hybridization of RNA isolated from the midguts of either control larvae or ecdysone-injected larvae (see Fig. 5B) revealed that both *rpr* and *hid* are induced in this tissue in response to ecdysone (data not shown).

#### DISCUSSION

Insect metamorphosis entails a radical reconstruction of the body plan, from a crawling larva to a highly motile and reproductively active adult fly. The massive destruction of obsolete larval tissues is an integral aspect of this transformation, allowing for their replacement by adult tissues and structures. In this study, we provide evidence that larval midgut and salivary gland histolysis is a steroid-triggered programmed cell death response that displays many of the hallmark features of

apoptosis. We also show that both positive and negative death regulators are expressed in patterns that correlate with the onset of larval cell death, and we present a model that integrates these genes into the ecdysone-triggered regulatory hierarchies that direct the early stages of *Drosophila* metamorphosis. These studies provide a foundation for understanding the molecular mechanisms by which a steroid signal is transduced to result in discrete stage- and tissue-specific programmed cell death responses during development.

### Larval midgut and salivary gland histolysis are steroid-triggered programmed cell death responses

In this study, we have focused on the destruction of two larval tissues during the onset of *Drosophila* metamorphosis – the midgut and salivary glands. These tissues represent two distinct temporal responses to ecdysone and undergo histolysis in different ways. Larval midgut histolysis is coupled to the growth and differentiation of the adult gut, such that the adult gut forms and encompasses the larval cells as they undergo apoptosis during prepupal development. In contrast, the destruction of the larval salivary gland occurs relatively suddenly, approximately 15 hours after the onset of midgut histolysis. The timing of salivary gland histolysis, several hours after the ecdysone pulse that triggers the prepupal-pupal transition, suggests that it is a hormone triggered event. This is consistent with the results of Aizenson and Zhimulev (1975) who demonstrated that the destruction of *Drosophila* larval salivary glands cultured in adult abdomens was dependent on the injection of ecdysone. Our results confirm and extend this study by demonstrating that larval salivary glands will initiate cell death when cultured in the presence of ecdysone (Fig. 5A). Similarly, larval midguts from animals injected with ecdysone undergo cell death, whereas midguts from control animals do not (Fig. 5B). These results indicate that the destruction of both the larval salivary glands and midgut are steroid-triggered programmed cell death responses.

Our studies also indicate that larval midgut and salivary gland histolysis proceeds through what appears to be a classic apoptotic response. The destruction of both tissues is foreshadowed by an intense and uniform nuclear acridine orange stain, indicative of the increased nuclear permeability that accompanies apoptosis (Fig. 2) (Abrams et al., 1993). The death of these tissues is also accompanied by nuclear DNA fragmentation, another characteristic of apoptosis (Fig. 3). Finally, larval midgut and salivary gland histolysis can be inhibited by expression of the anti-apoptotic baculovirus p35 protein (Fig. 4). Because p35 functions by directly inhibiting caspase activity (Bump et al., 1995; Xue and Horvitz, 1995), this result implicates caspases as essential components of the histolysis pathway. Taken together, these observations indicate that larval tissue histolysis proceeds as a classic apoptotic response. A similar conclusion has been made by Lockshin and Zakeri (1994) for the destruction of the labial gland during the onset of *Manduca* metamorphosis. It seems likely that the simultaneous destruction of other larval tissues, including the larval muscles, hindgut and larval neurons in the central nervous system, also represent steroid-triggered programmed cell death responses.

It is interesting to note that the cessation of feeding at the end of larval development is coupled to the subsequent histolysis of the larval midgut. In *Manduca*, a variety of behavioral

and developmental changes occur in response to a low titer ecdysone pulse that precedes the high titer hormone pulse that triggers pupation. These responses include the cessation of feeding, initiation of wandering, and pupal commitment of the epidermis (Dominick and Truman, 1985). Late third instar *Drosophila* larvae also stop feeding and wander in an ecdysone-dependent manner (Berreur et al., 1984). These responses not only allow the animal to move to a suitable place for pupariation, but also effectively clear the gut of food prior to its metamorphosis. It seems likely that this early response to ecdysone is critical for the subsequent histolysis of the gut, since any remaining food might complicate the destruction of this tissue.

### Induction of *rpr* and *hid* expression correlates with the onset of larval midgut and salivary gland programmed cell death

Two *Drosophila* death genes, *rpr* and *hid*, are expressed in a stage- and tissue-specific manner that implicates them as key regulators of larval tissue histolysis. These genes are induced at the onset of metamorphosis in whole animals and expressed throughout prepupal and early pupal development, reflecting the massive destruction of larval tissues that occurs at these stages (Fig. 6A). In isolated larval midguts and salivary glands, *rpr* and *hid* are coordinately induced in a stage-specific manner that foreshadows the death of these tissues – in the midguts of newly formed prepupae and in the salivary glands 12 hours later (Fig. 6B,C). Furthermore, *rpr* and *hid* induction precedes the death of salivary glands by 2–3 hours, identical to the delay seen in embryos (White et al., 1994; Grether et al., 1995). This correlation is not apparent in the midgut, where *rpr* and *hid* are expressed at high levels in newly formed prepupae, along with the onset of nuclear acridine orange staining. It is likely that this apparent synchrony is due to the inaccuracy of staging late third instar larvae. Each animal in the –4 hour population could be anywhere from 1–10 hours before pupariation (Andres and Thummel, 1994). It is thus possible that we could miss a response that occurs a few hours before puparium formation, making it difficult to determine the exact timing and order of death gene induction and apoptosis at this stage in development.

### The *diap2* anti-cell death gene may contribute to the regulation of larval salivary gland histolysis

Since the discovery of viral inhibitors of apoptosis (vIAPs) (Crook et al., 1993; Birnbaum et al., 1994), a family of *iap* genes have been identified in both *Drosophila* and humans (Hay et al., 1995; Rothe et al., 1995; Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996). The proteins encoded by these genes are characterized by N-terminal BIR repeats and a C-terminal RING zinc-finger motif, and are known to effectively block cell death in insects and mammals.

We have examined the expression of two *Drosophila iap* genes, *diap1* and *diap2*, during larval midgut and salivary gland histolysis. Interestingly, *diap2* is induced in mid-prepupal salivary glands and repressed in late prepupae, as *rpr* and *hid* are induced (Fig. 6C). Furthermore, the switch from *diap2* to *rpr* is triggered by ecdysone (Fig. 7), under conditions that lead to programmed cell death (Fig. 5). These observations suggest that salivary gland cell death is under both positive and negative regulation, and that the timing of histolysis is coordi-



nated by ecdysone. This model is consistent with our current understanding of the regulation of programmed cell death, in which negative regulators, such as *iaps* or *ced-9/Bcl-2* family members, function to balance death inducers, such as *rpr* and *hid* (Raff, 1992; Jacobson et al., 1997). The timing and regulation of *diap2* expression in the salivary gland provide an ideal system for testing the hypothesis that this gene functions to oppose the activity of death inducers in coordinating an apoptotic response during development.

### A model for the steroid-regulation of programmed cell death during *Drosophila* metamorphosis

The observation that *rpr* and *hid* induction immediately precedes the steroid-triggered destruction of the larval salivary glands provides us with an ideal opportunity to understand the hormonal regulation of programmed cell death. By identifying the factors that direct *rpr* and *hid* induction, we should be able to define a steroid-triggered regulatory hierarchy that leads to a programmed cell death response. Furthermore, because the death program has been conserved through evolution, it seems likely that the genetic pathways that we define for ecdysone-regulated cell death during insect metamorphosis will provide a foundation for understanding how hormones direct apoptosis during the development of other higher organisms.

Two ecdysone-inducible early genes could function as regulators of *rpr* and *hid* transcription during metamorphosis: the *Broad-Complex (BR-C)* and *E93*. The *BR-C* is induced directly by ecdysone during larval and prepupal development and encodes a family of zinc finger transcription factors (DiBello et al., 1991; Bayer et al., 1996). Genetic studies have shown that mutations in the *BR-C* result in a failure of larval midgut and salivary gland histolysis (Restifo and White, 1992). This observation argues that the *BR-C* may be essential for appropriate *rpr* and *hid* induction in these tissues. The *BR-C* is, however, induced by ecdysone at multiple stages of development, indicating that it can not be sufficient to direct the death response (Emery et al., 1994). Rather, we propose that the *BR-C* may function together with a stage-specific regulator to determine when death is induced. The *E93* early gene is an ideal candidate for encoding this stage-specific function (Baehrecke and Thummel, 1995). *E93* is induced by ecdysone in a stage-specific manner in the larval midgut and salivary gland, immediately preceding the histolysis of these tissues. Furthermore, although *E93* protein has no matches in the sequence databases, its nuclear localization suggests that it may function as a transcriptional regulator like the products of the *BR-C*. This is further supported by our ability to detect *E93* protein bound to specific sites in the polytene chromosomes (G. Lam, P. Reid, C. S. T., and E. H. B., unpublished results).

Combined, these observations lead to a model for the steroid regulation of larval tissue histolysis. We propose that *E93* is induced in a precise stage- and tissue-specific manner that foreshadows the death of larval cells. We further propose that *E93* and the *BR-C* function together in a regulatory hierarchy that directs the induction of *rpr* and *hid* as secondary-response target genes in larval tissues that are fated to die. This pathway could be similar to the interaction of the *BR-C* and *E74* transcription factors in directly regulating late gene transcription in the salivary glands (Urness and Thummel, 1995; Crossgrove et al., 1996). The integration of *diap2* into these regulatory hierarchies remains unclear, although it is possible that *diap2*

could be induced by the  $\beta$ FTZ-F1 mid-prepupal transcription factor (Woodard et al., 1994) and that *diap2* repression could require appropriate early gene function. A critical test of our model requires further phenotypic characterization of *BR-C* mutations and the isolation of mutations in *E93*, efforts that are currently underway.

Our studies of the ecdysone-regulation of *rpr* and *hid* transcription may also provide an opportunity to determine their functional significance during larval tissue histolysis. The fact that ectopic expression of either *rpr* or *hid* is sufficient to induce death throughout *Drosophila* development strongly suggests that the induction of these genes in larval tissues is of functional significance (Grether et al., 1995; Hay et al., 1995; White et al., 1996). Unfortunately, it is difficult to create somatic clones to test *rpr* and *hid* function in the larval midgut and salivary glands since there are only 2-3 mitotic divisions that precede the onset of endoreplication in the precursors of these tissues (Campos-Ortega and Hartenstein, 1985; Smith and Orr-Weaver, 1991). It is likely that clones resulting from these studies would lead to a high frequency of embryonic lethality (White et al., 1994). Functional studies are further complicated by the apparent redundancy among the three *Drosophila* death genes. If, however, *rpr* and *hid* are not induced in *BR-C* or *E93* mutants, we can ask if ectopic *rpr* or *hid* expression is sufficient to restore death in a *BR-C* or *E93* mutant background. The ability of these genes to direct death under these conditions would argue that the death machinery is in place in these mutants, and that the simple failure of *rpr* and *hid* expression is sufficient to prevent the destruction of these tissues.

In closing, it is interesting to note that *Xenopus* metamorphosis also involves the massive destruction of larval tissues in response to a systemic hormonal signal. Like the *Drosophila* larval midgut, the tadpole intestine undergoes simultaneous cell death and adult cell proliferation to form the adult intestine, reflecting the change from a herbivorous to a carnivorous diet (McAvoy and Dixon, 1977; Yoshizato, 1996). This remodeling is triggered by thyroid hormone and is accompanied by dramatic changes in gene expression (Shi and Brown, 1993). In addition, the tadpole tail is rapidly and completely resorbed during metamorphosis, analogous to *Drosophila* larval salivary gland histolysis. This process also requires thyroid hormone and involves the specific induction and repression of primary- and secondary-response genes in the tail (Brown et al., 1996). Some of these genes are shared in common with the intestinal remodeling program, consistent with a role for cell death in both processes. It will be interesting to determine if death pathways similar to those described in mammalian cells are activated by thyroid hormone during *Xenopus* metamorphosis. Furthermore, given that many death regulators have been conserved from worms to humans, it will be interesting to determine if there are common features in the hormonal regulation of programmed cell death during insect and frog metamorphosis.

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