Developmental Biology **250,** 101–111 (2002)
doi:10.1006/dbio.2002.0784



# during *Drosophila* Development

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**Steroid hormones trigger dynamic tissue changes during animal development by activating cell proliferation, cell differentiation, and cell death. Here we characterize steroid regulation of changes in midgut structure during the onset of *Drosophila* metamorphosis. Following an increase in the steroid 20-hydroxyecdysone (ecdysone) at the end of larval development, future adult midgut epithelium is formed, and the larval midgut is rapidly destroyed. Mutations in the steroid-regulated genes *BR-C* and *E93* differentially impact larval midgut cell death but do not affect the formation of adult midgut epithelia. In contrast, mutations in the ecdysone-regulated *E74A* and *E74B* genes do not appear to perturb midgut development during metamorphosis. Larval midgut cells possess vacuoles that contain cellular organelles, indicating that these cells die by autophagy. While mutations in the *BR-C*, *E74*, and *E93* genes do not impact DNA degradation during this cell death, mutations in *BR-C* inhibit destruction of larval midgut structures, including the proventriculus and gastric caeca, and *E93* mutants exhibit decreased formation of autophagic vacuoles. Dying midguts express the *rpr*, *hid*, *ark*, *dronc*, and *crq* cell death genes, suggesting that the core cell death machinery is involved in larval midgut cell death. The transcription of *rpr*, *hid*, and *crq* are altered in *BR-C* mutants, and *E93* mutants possess altered transcription of the caspase *dronc*, providing a mechanism for the disruption of midgut cell death in these mutant animals. These studies indicate that ecdysone triggers a two-step hierarchy composed of steroid-induced regulatory genes and apoptosis genes that, in turn, regulate the autophagic death of midgut cells during development.** 

***Key Words:*** **steroid; ecdysone; programmed cell death; apoptosis; autophagy; development; metamorphosis; *Drosophila*.**

## INTRODUCTION

Steroid hormone signaling plays an important role in the maintenance of homeostasis in higher eukaryotes. During development, steroids trigger cell proliferation, cell differentiation, and cell death. Several steroid hormones are involved in the regulation of higher vertebrate development. Testosterone and estrogen, for example, regulate the development of sex-specific structures. During mammary development, testosterone triggers the destruction of mammary cells in males (Kratochwil and Schwartz, 1976). In female mammary tissue, estrogen appears to be involved in the maintenance of milk protein synthesis and inhibition of luminal epithelial cell death. While invertebrate organisms utilize steroids to regulate developmental changes, a single steroid appears to be responsible for much of this signaling. This suggests that the genetic regulatory hierarchy that is activated by steroids in invertebrates is responsible for the specificity of the cell response. In insects, the steroid 20-hydroxyecdysone (ecdysone) triggers cell proliferation, cell differentiation, and cell death (Riddiford, 1993). The mechanisms underlying steroid signaling have been extensively studied in the fruit fly, *Drosophila melanogaster*, because of the strength of this system for molecular genetics.

*Drosophila* metamorphosis is triggered by an increase in ecdysone at the end of third larval instar development (Riddiford, 1993). This increase in hormone titer triggers the formation of a prepupa. The ecdysone titer drops to a low level in 4- to 6-h prepupae, and rises in 10- to 12-h prepupae, triggering the formation of a pupa. These two consecutive pulses of hormone activate stage- and cell-specific developmental responses (Baehrecke, 2000). The increase in ecdysone at the end of the third instar induces imaginal disc evagination, a dramatic change in larval salivary gland gene transcription, and larval midgut cell death. In contrast, the subsequent increase in ecdysone at

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0012-1606/02 $35.00

the end of prepupal development triggers salivary gland programmed cell death. Thus, larval midguts and salivary glands exhibit stage-specific cell death responses to ecdysone. Midguts and salivary glands both exhibit markers of programmed cell death following these increases in ecdysone, including nuclear staining by acridine orange and DNA fragmentation (Jiang *et al.*, 1997). In addition, expression of baculovirus p35 inhibits destruction of both of these tissues, indicating that caspases are involved in the destruction of the midgut and salivary glands. Furthermore, the cell death genes *reaper* (*rpr*) and *head involution defective* (*hid*) increase in transcription preceding both larval midgut and salivary gland programmed cell death.

Salivary glands have served as a useful model for understanding steroid regulation of cell death because of several attributes including the synchronized death of cells and the well-characterized genetic signaling hierarchy in this tissue. The nuclear receptor genes *EcR* and *usp* encode components of the hormone receptor complex (Thomas *et al.*, 1993; Yao *et al.*, 1992). The ecdysone receptor complex and the competence factor βFTZ-F1 regulate the transcription of *BR-C*, *E74*, and *E93* (Broadus *et al.*, 1999; Woodard *et al.*, 1994). The *EcR*, *βFTZ-F1*, *BR-C*, *E74*, and *E93* genes all appear to function in larval salivary gland programmed cell death (Broadus *et al.*, 1999; Jiang *et al.*, 2000; Lee *et al.*, 2000; Restifo and White, 1992; Robinow *et al.*, 1993; Truman *et al.*, 1994). While *EcR*, *βFTZ-F1*, *BR-C*, and *E74* function in processes other than cell death (Bender *et al.*, 1997; Broadus *et al.*, 1999; Fletcher *et al.*, 1995; Restifo and White, 1992), *E93* appears to function more specifically in cell death (Lee *et al.*, 2000). *BR-C*, *E74*, and *E93* impact the transcription of programmed cell death genes, including *rpr*, *hid*, *crq*, *ark*, and the caspase *dronc*, during salivary gland destruction (Jiang *et al.*, 2000; Lee *et al.*, 2000). The increased transcription of these core cell death genes argues that these cells die by apoptosis, but salivary glands exhibit the morphology of cells that die by autophagy (Lee and Baehrecke, 2001).

The death of the *Drosophila* larval midgut differs from larval salivary glands. Ecdysone triggers midguts to die at a stage when salivary glands synthesize and secrete polypeptide glue. In addition, future adult midgut cells are dispersed in the larval midgut epithelium, while adult salivary gland cells are restricted in one ring of cells (Robertson, 1936). Thus, adult midgut cells appear to utilize the dying larval midgut as a substrate that they develop around, whereas the larval salivary gland appears to be dispensible for adult salivary gland formation. The relationship between adult and larval midgut cells presents a level of complexity that does not appear to be present in salivary glands. Finally, while expression of the inhibitor of caspases p35 prevents proper destruction of midguts, it is not clear whether these cell die by apoptosis or autophagy.

Here we analyze changes in midgut structure, gene transcription, and the impact of mutations on midgut development during the onset of *Drosophila* metamorphosis. The future adult midgut begins to form in new prepupae, and envelops the degenerating larval midgut by 2 h following puparium formation. The proventriculus and gastric caeca of the larval midgut decrease in size in the initial 4 h of prepupal development, and are unrecognizable as distinct structures by 6 h after puparium formation. Midgut cells exhibit increased numbers of cytoplasmic inclusions, including vacuoles containing organelles, crystalline inclusions, and myelin-like figures, at the onset of metamorphosis, indicating that midguts die by autophagy. Mutations in the *BR-C*, *E93*, *E74A*, and *E74B* genes do not appear to impact either the formation of the future adult midgut or the dramatic shortening of the midgut that occurs at this stage in development. This suggests that future adult cells are responsible for the change in midgut length, since mutations in *BR-C* and *E93* impact larval midgut cell death. Mutations in *BR-C* prevent the destruction of the larval proventriculus and gastric caeca, and *E93* mutants exhibit defects in the degradation of the midgut cell cytosol, but *E74A* and *E74B* do not exhibit any defects in midgut cell death. The cell death genes *rpr*, *hid*, *crq*, *ark*, and *dronc* increase in transcription in dying midguts, and mutations in *BR-C* result in altered *rpr*, *hid*, and *crq* RNA levels during midgut cell death, while *E93* mutants have altered levels of the caspase *dronc*. These studies indicate that larval midguts and salivary glands utilize similar, yet distinct, genetic mechanisms during steroid regulation of programmed cell death.

## MATERIALS AND METHODS

### *Histology*

Canton S wild-type and *BR-C* ($2Bc^2/Y$), *E74A* ($E74A^{P[neo]}/Df(3L)st\text{-}81k19$), *E74B* ($E74B^{DL\text{-}1}/Df(3L)st\text{-}81k19$), and *E93* ($E93^1/Df(3R)93F^{x2}$) mutant animals were staged in hours after puparium formation at 25°C. For paraffin sections and light microscopy, animals were fixed, embedded, sectioned, stained as previously described (Restifo and White, 1991), and analyzed by using a Zeiss Axiophot II microscope. For electron microscopy, midguts were dissected from staged animals, fixed in 3% glutaraldehyde/0.2% tannic acid in 0.1 M Mops buffer (pH 7.0) for 8 h, 3% glutaraldehyde/1% paraformaldehyde in 0.1 M Mops buffer (pH 7.0) for 16 h at 4°C, postfixed in 1% osmium tetroxide for 1 h, embedded in Spurr's resin, sectioned, and analyzed with a Zeiss EM 10 transmission electron microscope.

### *Quantification of Midgut Length*

Canton S wild-type and *BR-C* ($2Bc^2/Y$), *E74A* ($E74A^{P[neo]}/Df(3L)st\text{-}81k19$), *E74B* ($E74B^{DL\text{-}1}/Df(3L)st\text{-}81k19$), and *E93* ($E93^1/Df(3R)93F^{x2}$) mutant midguts were dissected in phosphate-buffered saline from animals staged in hours after puparium formation at 25°C. The length of unfixed midguts (distance between the anterior most point where the gastric caeca join the gut and the anterior most point where the Malpighian tubules join the gut) was immediately quantified by using an ocular micrometer on a Zeiss SV11 microscope.

### *Cell Death Detection*

Canton S wild-type and *BR-C* ($2Bc^2/Y$), *E74A* ($E74A^{P[neo]}/Df(3L)st\text{-}81k19$), *E74B* ($E74B^{DL\text{-}1}/Df(3L)st\text{-}81k19$), and *E93* ($E93^1/Df(3R)93F^{x2}$) mutant animals were staged in hours after puparium formation at 25°C, fixed, embedded in paraffin, and sectioned as previously described (Restifo and White, 1991). Paraffin sections were subjected to the TUNEL assay, as described (White *et al.*, 1994), and counterstained with eosin Y.

### *Northern Blot Hybridization*

Midguts were dissected from wild-type Canton S staged in hours relative to puparium formation at 25°C in order to determine the temporal pattern of cell death gene transcription. To determine whether *BR-C* ($2Bc^2/Y$) and *E93* ($E93^1/Df(3R)93F^{x2}$) mutants influence the transcription of cell death genes, midguts were collected from mutant animals staged in hours relative to puparium formation at 25°C as described (Andres and Thummel, 1994). RNA was extracted, electrophoresed, transferred to nylon membranes, and hybridized with radiolabeled probes following previously described methods (Baehrecke and Thummel, 1995). Control wild-type and mutant Northern blots were hybridized at the same time with probes to detect the cell death genes *rpr*, *hid*, *ark*, *dronc*, *crq*, and *rp49* as a loading and transfer control. Exposures of control and mutant Northern blots were normalized based on the time required for equal detection of *rp49*.

## RESULTS

### *Larval Midgut Is Destroyed While the Adult Midgut Forms during Prepupal Development*

The morphology of midguts was examined at the onset of metamorphosis to provide a framework for studies of genetic regulation of larval midgut cell death. Wild-type Canton S were staged in hours following puparium formation, fixed, embedded in paraffin, sectioned, and stained. New prepupae possess a larval esophagus, proventriculus, gastric caeca, and midgut structures, and exhibit no signs of larval cell death or adult midgut formation at this resolution (Fig. 1A, $n = 10$). Two hours after puparium formation, the proventriculus, gastric caeca, and larval midgut are surrounded by an adult epithelium (Fig. 1B, $n = 10$). In 4-h prepupae, the proventriculus and gastric caeca appear to compress toward the larval midgut, and the larval epithelium becomes convoluted, causing a large space in the larval lumen (Fig. 1C, $n = 10$). Six hours after puparium formation, the proventriculus and gastric caeca can no longer be distinguished, and the larval midgut becomes further condensed (Fig. 1D, $n = 10$). The larval midgut is extremely condensed 12 h after puparium formation, and the adult and larval epithelia have separated such that a defined adult lumen exists (Fig. 1E, $n = 10$).

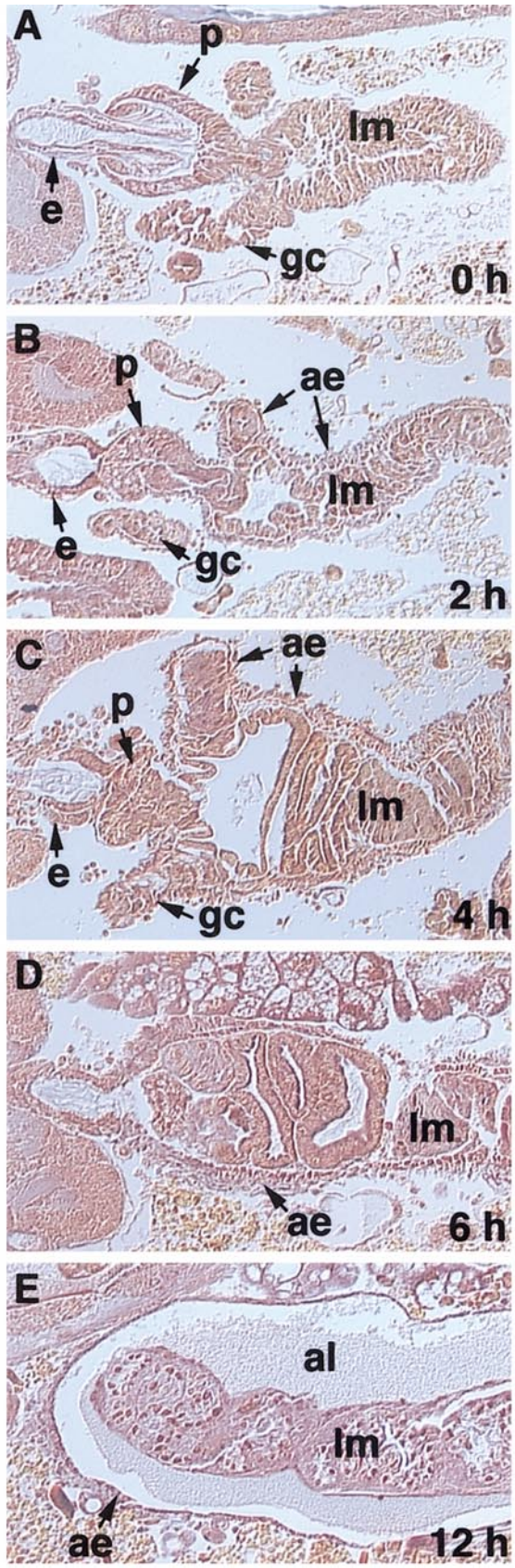
### *Mutations in Ecdysone-Regulated Genes Impact Larval Midgut Cell Death*

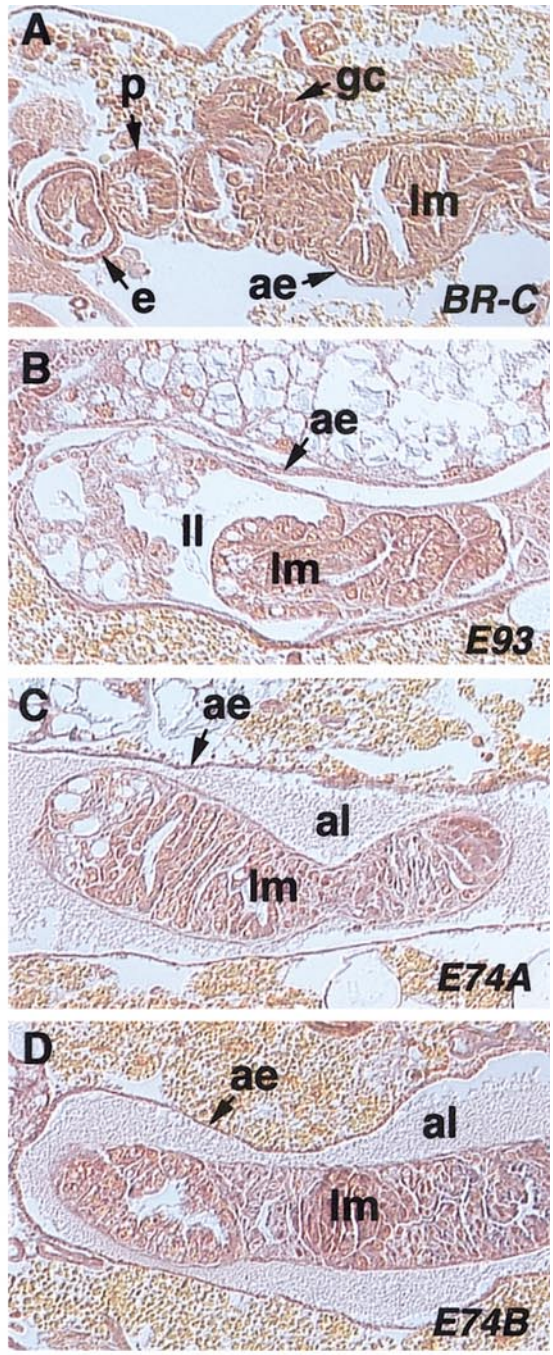
The death of larval midgut cells coincides with the increase in ecdysone that triggers puparium formation, and premature elevation of the ecdysone titer in third instar larvae is sufficient to ectopically induce cell death in larval midguts (Jiang *et al.*, 1997). In addition, mutations in the ecdysone receptor and the ecdysone-regulated primary response gene *BR-C* prevent proper destruction of larval midguts (Bender *et al.*, 1997; Hall and Thummel, 1998; Restifo and White, 1992). We investigated the role of the ecdysone-regulated primary response genes *BR-C*, *E93*, and *E74* in larval midgut destruction, since these genes regulate steroid-activated destruction of larval salivary glands (Jiang *et al.*, 2000; Lee and Baehrecke, 2001).

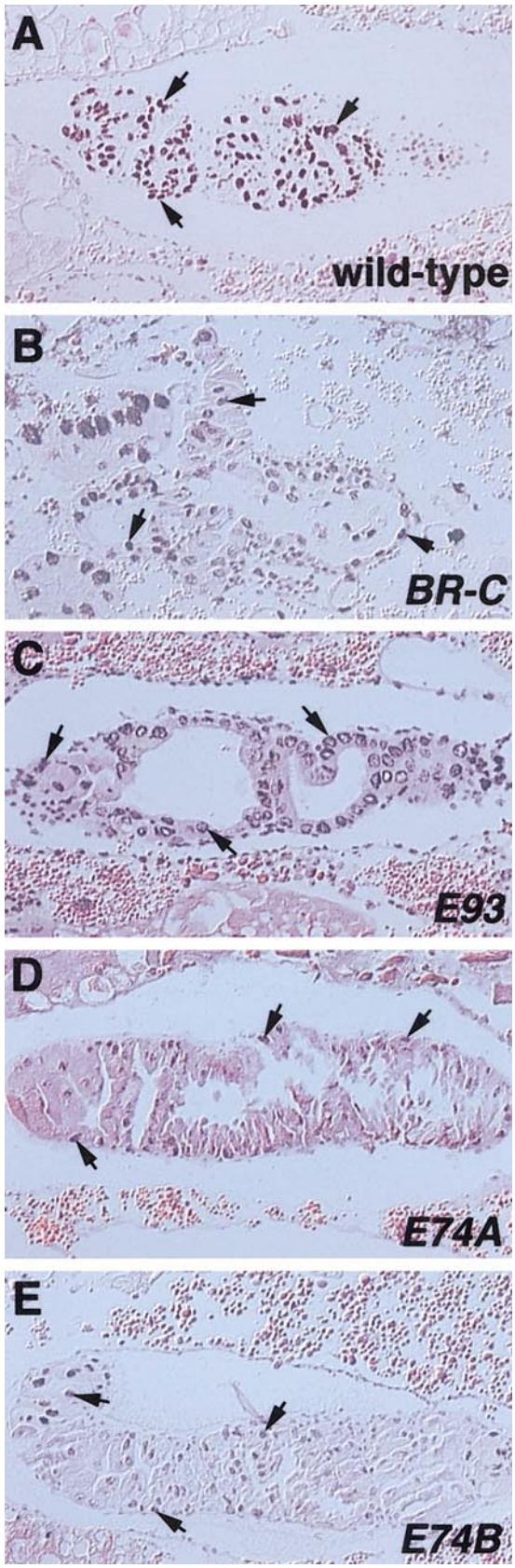
To analyze the destruction of mutant larval midguts, animals were staged at pupal head eversion, fixed, embedded in paraffin, sectioned, and analyzed by light microscopy for defects in midgut structure. Head eversion was selected as the stage for analyses as this is 12 h after midgut destruction is initiated and the larval midguts of control animals are fully compressed at this time (Fig. 1E). *BR-C*

**FIG. 1.** Midguts exhibit dynamic morphological changes during the onset of metamorphosis. Wild-type Canton S animals were staged relative to puparium formation, fixed, embedded in paraffin, sectioned, and midguts were analyzed by light microscopy. (A) Larval midguts (lm) of new prepupae possess a larval esophagus (e), proventriculus (p), and gastric caeca (gc). (B) Structures of the larval midgut including the proventriculus (p) and gastric caeca (gc) are surrounded by an adult epithelium (ae) 2 h after puparium formation. (C) Four hours after puparium formation, the proventriculus (p) and gastric caeca (gc) appear to compress, the larval midgut epithelium becomes convoluted, and a large space forms in the larval lumen. (D) The proventriculus and gastric caeca are not observed 6 h after puparium formation. (E) Twelve hours after puparium formation, the larval midgut is extremely condensed and the adult (ae) and larval epithelia have separated such that a defined adult lumen (al) exists.

**FIG. 2.** Steroid-regulated genes regulate larval midgut destruction, but not adult midgut formation. Mutant animals were staged at the onset of pupation (future adult head eversion), fixed, embedded in paraffin, sectioned, and analyzed by light microscopy. (A) *BR-C* mutants ($2Bc^2/Y$) possess larval midgut (lm) structures, including a larval esophagus (e), proventriculus (p), and gastric caeca (gc), and are surrounded by an adult epithelium (ae). (B) *E93* ($E93^1/Df(3R)93F^{x2}$) mutant midguts have a convoluted larval midgut (lm) epithelium and a large space in the larval lumen (ll) and are surrounded by an adult epithelium (ae). (C) *E74A* mutant ($E74A^{P[neo]}/Df(3L)st\text{-}81k19$) larval midguts (lm) are extremely condensed and the adult (ae) and larval epithelia have separated such that a defined adult lumen (al) exists. (D) *E74B* mutant ($E74B^{DL\text{-}1}/Df(3L)st\text{-}81k19$) larval midguts (lm) are also extremely condensed, and the adult (ae) and larval epithelia have separated such that a defined adult lumen (al) exists.






($2Bc^2$) mutants have the strongest phenotype and always possess some remnants of the larval proventriculus and gastric caeca (Fig. 2A, $n = 10$). While larval midgut destruction does not occur properly in *BR-C* mutants, the adult epithelium is formed and the midgut appears to be arrested at a stage that is similar to the midgut of wild-type animals 2–4 h following puparium formation (Figs. 1B, 1C, and 2A). *E93* mutants always form an adult epithelium, and the larval proventriculus and gastric caeca are destroyed (Fig. 2B, $n = 10$). However, larval midgut compaction was never observed in *E93* mutants as indicated by the large space in the larval lumen (Fig. 2B). *E93* mutant larval midguts appear to be arrested at a stage of destruction that is similar to the midgut of wild-type animals 4–6 h following puparium formation (Figs. 1C, 1D, and 2B). *E74A* and *E74B* mutants also form an adult midgut epithelium, and the larval proventriculus and gastric caeca are destroyed (Figs. 2C and 2D, $n = 10$). Larval midgut compaction seems to occur to a greater extent in *E74A* and *E74B* mutants than in *E93* mutants, as *E74A* and *E74B* mutants develop a large space between the larval and adult midgut epithelia (Figs. 2B–2D). Thus, *E74A* and *E74B* mutant larval midguts appear to be arrested at a stage of destruction that is similar to the midgut of wild-type animals 6–12 h following puparium formation (Figs. 1D, 1E, 2C, and 2D) and do not appear to impact larval midgut destruction.

The larval midgut shrinks dramatically during the first 6 h of pupariation when these cells are dying (Jiang *et al.*, 1997), suggesting that midgut shortening may be related to larval cell death. In order to quantify the relationship between the change in midgut length, cell death, and mutants that impact larval midgut destruction, we measured the length of midguts at puparium formation and head eversion. Wild-type Canton S midguts decrease from 7.80 to 1.60 mm in length, or 85%, during this interval (Table 1). While *BR-C*, *E93*, *E74A*, and *E74B* mutant larval midguts all decrease in size between puparium formation and head eversion, this shrinking varies (Table 1). *BR-C* mutant midguts decrease from 7.57 to 2.50 mm (65%) in length. *E93* midguts decrease from 6.38 to 1.64 mm (74%) between puparium formation and head eversion. Midguts of *E74A* mutants decrease from 6.55 to 1.21 mm (81%), while *E74B* mutants change from 5.97 to 1.62 mm (73%) in length. Therefore, midgut shortening likely relies on the newly formed adult midgut epithelium, as midguts shorten

**FIG. 3.** Mutations in steroid-regulated genes do not alter midgut DNA fragmentation. Wild-type Canton S and mutant animals were staged at the onset of pupation (future adult head eversion), fixed, embedded in paraffin, sectioned, processed to analyze DNA fragmentation using the TUNEL assay, and analyzed by light microscopy. (A) Wild-type, (B) *BR-C* ($2Bc^2/Y$), (C) *E93* ($E93^1/Df(3R)93F^{x2}$), (D) *E74A* ($E74A^{P[neo]}/Df(3L)st\text{-}81k19$), and (E) *E74B* ($E74B^{DL\text{-}1}/Df(3L)st\text{-}81k19$) larval midguts all possess dark TUNEL-positive nuclei (arrows).

**TABLE 1**
Changes in Midgut Length during the Onset of Metamorphosis

| Genotype | White prepupae | | Head everted | | Percent change |
|---|---|---|---|---|---|
| | *n* | mm (mean ± SD) | *n* | mm (mean ± SD) | |
| Canton S | 11 | 7.80 ± 0.60 | 12 | 1.60 ± 0.12 | 85 |
| $2Bc^{2}/Y$ | 12 | 7.57 ± 0.48 | 17 | 2.50 ± 0.45 | 67 |
| $E93^{1}/Df(3R)93F^{X2}$ | 10 | 6.38 ± 0.58 | 10 | 1.64 ± 0.12 | 74 |
| $E74A^{P(neo)}/Df(3L)st^{8[k]9}$ | 13 | 6.55 ± 0.72 | 13 | 1.21 ± 0.18 | 82 |
| $E74B^{DL-1}/Df(3L)st^{81k19}$ | 12 | 5.97 ± 0.44 | 10 | 1.62 ± 0.09 | 73 |

in *BR-C* and *E93* mutants that prevent proper destruction of larval cells (Fig. 2).

DNA fragmentation accompanies the destruction of larval midguts (Jiang *et al.,* 1997). The TUNEL procedure was used to determine whether mutations in ecdysone-regulated genes prevent DNA fragmentation in larval midgut cells. Wild-type Canton S and mutant animals were staged at head eversion, fixed, embedded in paraffin, sectioned, and analyzed for the presence or absence of DNA fragmentation (Fig. 3, $n = 5$–10 midguts/genotype). Canton S possess compacted midguts and fragmented DNA at head eversion (Fig. 3A). While *BR-C* mutants have persistent larval structures, including gastric caeca, every larval midgut cell nucleus of these mutants appeared to be labeled, indicating that they possess fragmented DNA (Fig. 3B). Similarly, the nuclei of *E93*, *E74A*, and *E74B* mutant midguts were all labeled following the TUNEL procedure (Figs. 3C–3E). These data suggest that larval midgut cells do not die by apoptosis, since mutations in the *BR-C* and *E93* genes prevent destruction of midgut cells, and the midgut cells of these mutants possess fragmented DNA.

### *Larval Midgut Cells Are Destroyed by Autophagy, and E93 Mutants Fail to Form Proper Autophagic Structures*

*Drosophila* larval midguts exhibit markers of apoptosis immediately prior to destruction, including DNA fragmentation and nuclear staining by acridine orange, as well as increased transcription of the proapoptotic genes *rpr* and *hid* (Jiang *et al.,* 1997). While mutations in the *BR-C* and *E93* genes prevent destruction of midgut cells, the midgut cells of these mutants possess fragmented DNA, suggesting that they do not die by apoptosis (Figs. 2 and 3). Thus, we utilized transmission electron microscopy to analyze cell structure during cell death of midguts. Late third instar larval midguts possess microvilli facing the lumen, large nuclei with banded polytene chromosomes, and intact mitochondria in the cytoplasm (Fig. 4A, $n = 3$). At this stage, very few indications of cell death exist, although we do observe small numbers of early stage autophagic vacuoles and swirls of rough endoplasmic reticulum (Fig. 4B), which is one of the mechanisms by which autophagic vacuoles are formed (Dunn, 1990). Larval midguts of new prepupae have microvilli facing the lumen, intact nuclei, and the cytoplasm has increased numbers of autophagic vacuoles and appears to possess more spaces than in late third instar larvae (Fig. 4C, $n = 3$). Vacuoles that contain structures, including organelles such as mitochondria and crystalline inclusions, are abundant in the larval midguts of new prepupae (Fig. 4D) and indicate that these cells die by autophagy. Two hours following puparium formation, the forming adult midgut is apparent, and the larval midgut cytoplasm possesses an increased number of vacuoles containing organelles, indicating that autophagy has progressed (Figs. 4E and 4F, $n = 3$). Larval midguts of 4-h prepupae appear to exhibit an increase in the number of nuclei per area examined, which is likely due to the compression of this structure (Fig. 4G, $n = 3$). Large numbers of crystalline inclusions were observed in the cytoplasm of larval midguts in 4-h prepupae (Figs. 4G and 4H). The proximity of nuclei increases and autophagic structures are abundant in larval midguts 6 h after puparium formation (Fig. 4I, $n = 3$). Twelve hours after puparium formation, the cytoplasm of larval midguts appears more condensed as fewer spaces are observed, and numerous autophagic structures, including myelin-like membrane swirls, were detected (Figs. 4J and 4K, $n = 3$). These data indicate that larval midguts die by autophagy and do not exhibit morphological characteristics of apoptosis.

Larval midgut cells possess vacuoles that contain cytosolic structures, such as mitochondria, indicating that these cells die by autophagy. Thus, we tested whether mutations in the *BR-C*, *E93*, *E74A*, and *E74B* prevent the destruction of the cytoplasm. The midgut cells of *BR-C* mutants exhibit variable cytoplasmic staining: some cells are extremely osmophylic, while others are not stained as dark (Fig. 5A). *BR-C* mutant midgut cells contain intact mitochondria (Fig. 5B) and did not exhibit obvious alterations in cytosolic structures from midguts of third instar larvae other than containing large spaces (Fig. 5A). In contrast, *E93* mutant midguts possess numerous cells that contain swollen mitochondria, and many of these organelles rupture (Figs. 5C and 5D). Not all *E93* mutant midgut cells completely lack autophagic structures, however, as some mitochondria are enclosed by membranes (Fig. 5C). The midguts of *E74A* and *E74B* mutants contain intact mitochondria that are ob-

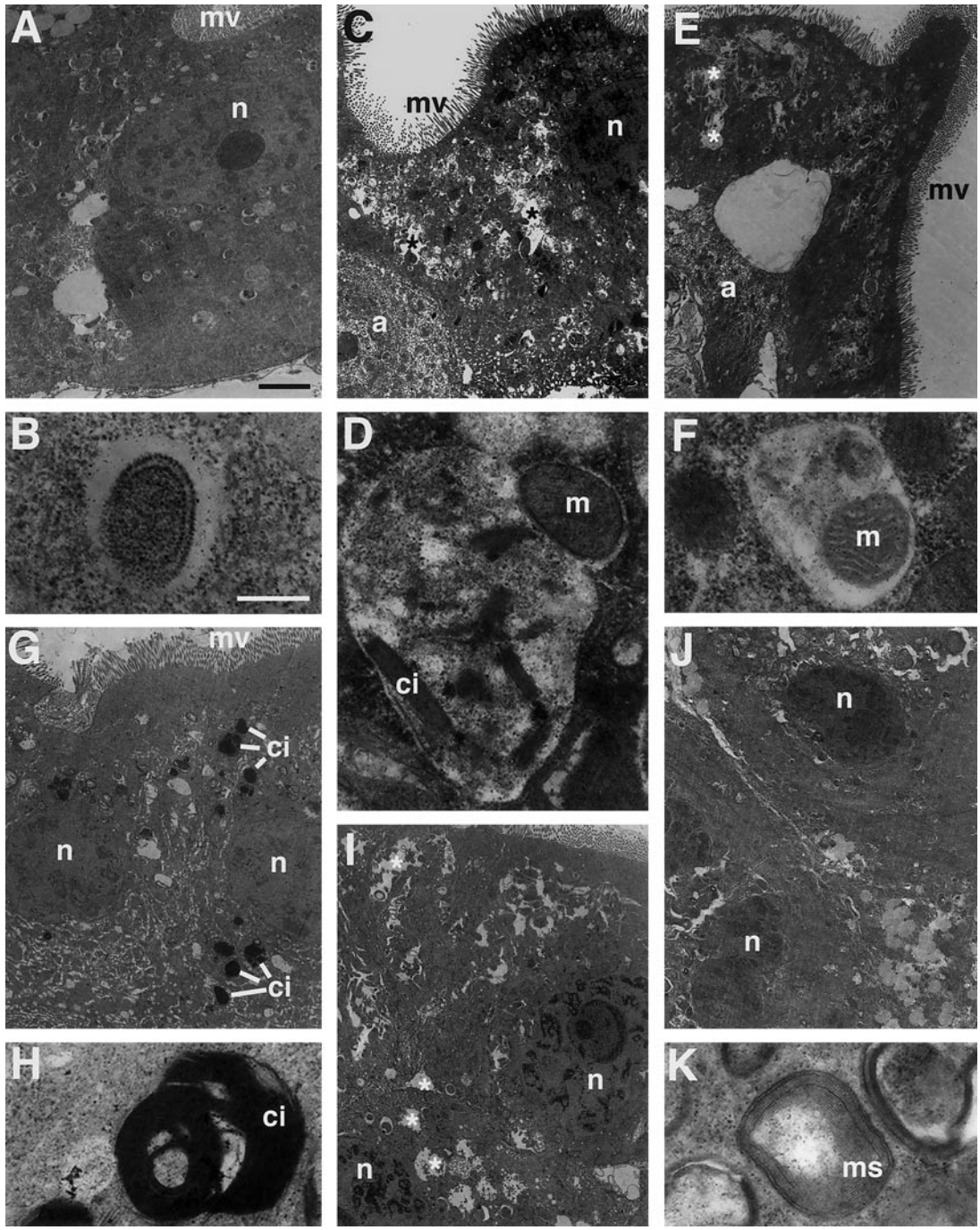



**FIG. 4.** Larval midgut destruction occurs by autophagy. Midguts were dissected from wild-type Canton S animals staged relative to puparium formation, fixed, sectioned, and analyzed by transmission electron microscopy. (A) Midguts of late third instar larvae have microvilli (mv) facing the lumen, large nuclei (n) with polytene chromosomes, and intact mitochondria. (B) Midguts of late third instar larvae have small numbers of swirls of rough endoplasmic reticulum in the cytosol. (C, D) Midguts of new prepupae have microvilli (mv) facing the lumen, intact nuclei (n), increased numbers of autophagic vacuoles in the cytosol that contain crystalline inclusions (ci), and mitochondria (m), and appear to possess more spaces (asterisks) than in late third instar larvae. Future adult (a) midgut cells still exist as isolated individual cells. (E, F) The forming adult midgut is apparent 2 h following puparium formation (a), while the larval midgut still has microvilli, and the cytoplasm possesses an increased number of vacuoles containing organelles including mitochondria. (G, H) Four-hour prepupal midguts appear to exhibit an increase in the number of nuclei (n) per area examined, and numerous crystalline inclusions (ci) in the cytosol. (I) Numerous nuclei (n), autophagic structures, and spaces (asterisks) are present in larval midguts 6 h after puparium formation. (J, K) The cytoplasm of larval midguts appears more condensed possessing fewer spaces 12 h after puparium formation, and numerous autophagic structures, including myelin-like membrane swirls (ms), are present. Scale bar in (A), 3 $\mu$m; (B) 0.3 $\mu$m. (A, C, E, G, I, J) are the same magnification, while (B, D, F, H, K) are the same magnification.

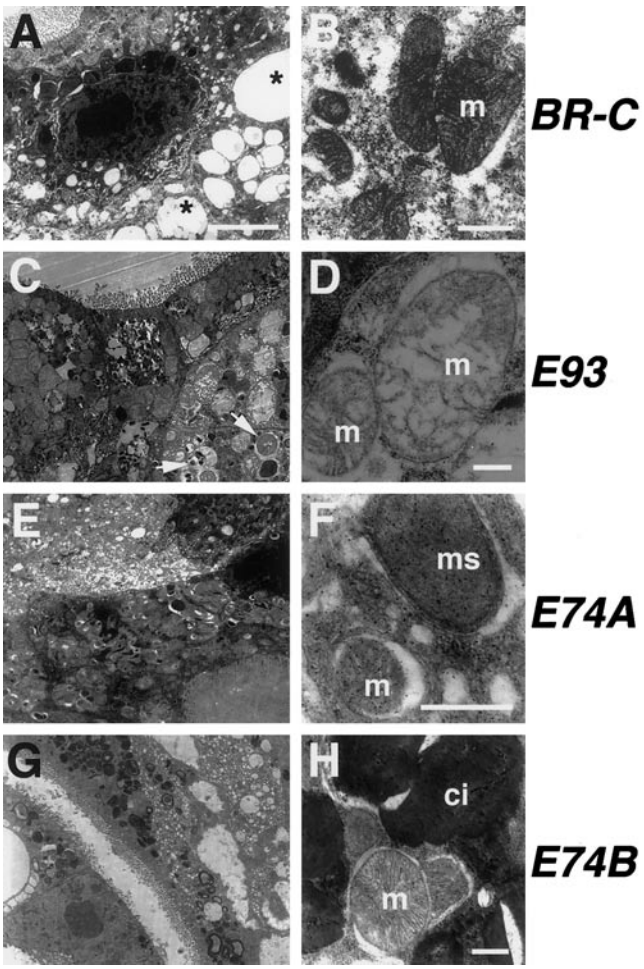



**FIG. 5.** Mutations in steroid-regulated genes impact midgut destruction by autophagy. Mutant animals were staged at the onset of pupation (future adult head eversion), midguts were dissected, fixed, sectioned, and analyzed by transmission electron microscopy. (A, B) *BR-C* mutant ($2Bc^2/Y$) larval midgut cells possess variable staining, large spaces (asterisks), and intact mitochondria (m). (C, D) *E93* mutant ($E93^1/Df(3R)93F^{x2}$) larval midgut cells possess large numbers of swollen and ruptured mitochondria (m), even though early stage autophagic vacuoles form in some cells (arrows). (E, F) *E74A* mutant ($E74A^{P[neo]}/Df(3L)st\text{-}81k19$) and (G, H) *E74B* mutant ($E74B^{DL\text{-}1}/Df(3L)st\text{-}81k19$) larval midgut cells appear to die normally and have numerous autophagic structures, including mitochondria (m) in vacuoles, crystalline inclusions (ci), and myelin-like membrane swirls (ms). Scale bar in (A) 5 $\mu$m; (B, D, F, H) 0.5 $\mu$m. (A, C, E, G) are the same magnification.

served in autophagic vacuoles (Figs. 5F and 5H). While *BR-C* mutants exhibit defects in the destruction of gross larval structures and *E93* mutants exhibit defects in the destruction cytosolic midgut structures (such as mitochondria), no similar defects were observed in either *E74A* or *E74B* mutant midguts, which possess numerous normal autophagic structures (Figs. 5E–5H).

### *Mutations in Steroid-Regulated Genes Impact Transcription of Apoptosis Genes during Larval Midgut Cell Death*

Expression of the caspase inhibitor p35 prevents midgut cell death (Jiang *et al.*, 1997). Since caspases are generally considered proteases that regulate apoptosis, we wanted to determine whether caspases and other cell death regulators are transcribed in midguts that die by autophagy. While it is known that *rpr* and *hid* are induced in dying midguts (Jiang *et al.*, 1997), it is not known whether other candidate cell death regulators are induced in these cells. Therefore, developmental Northern blots were prepared from wild-type midguts at stages preceding and during cell death. Transcription of *rpr*, *hid*, *ark*, *dronc*, and *crq* increases in wild-type animals following the late larval pulse of ecdysone that triggers larval midgut cell death (Fig. 6). Since mutations in the *BR-C* and *E93* genes prevent proper destruction of larval midguts (Figs. 2 and 5), Northern blots were prepared from midguts of these mutants at stages preceding and during cell death. *BR-C* $2Bc^2$ mutants have altered transcription of *rpr*, *hid*, and *crq*, but do not impact the transcription of *ark* and *dronc* (Fig. 6). In contrast, *E93* mutants possess altered transcription of *dronc*, but did not change the transcript levels of the other cell death genes known to be expressed in dying midguts (Fig. 6). Although midguts die by autophagy, they transcribe core apoptosis regulators during this cell death, and mutants that prevent autophagy alter transcription of apoptosis genes.

## DISCUSSION

Studies of developing vertebrate and invertebrate organisms indicate that at least two forms of programmed cell death have been conserved in diverse animals (Clarke, 1990; Schweichel and Merker, 1973). Apoptotic cell death is usually observed when isolated cells die, while autophagic cell death seems to occur when groups of cells or entire tissues die. Studies of cell lines indicate that the biochemical mechanisms underlying programmed cell death are extremely similar in different cell types. However, the derived nature and morphological similarities of these apoptotic cells indicate that such systems are not ideal for the identification of possible differences in dying cells. The cell deaths that occur *in vivo* are morphologically diverse, and identifying the mechanisms that regulate physiological programmed cell death will lead to a better understanding of how perturbation of this fundamental cellular process leads to aberrations in animal growth and development. We have characterized programmed cell death of larval midguts in *Drosophila* as a model for steroid-triggered cell death during development. Dying midguts and salivary glands both appear to utilize components of the core apoptotic machinery, even though these cells exhibit morphological characteristics of autophagic cell death. However, these similar steroid-regulated cell deaths utilize distinct mechanisms for their stage-specific activation. Thus, these studies

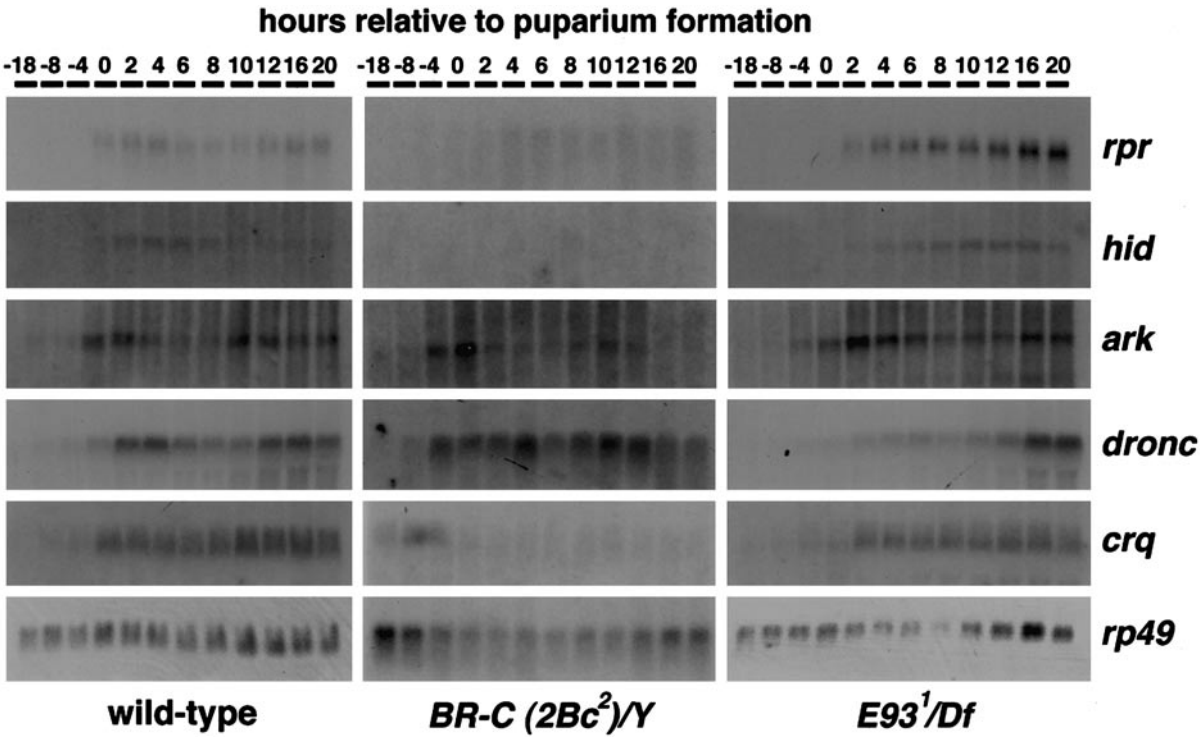



**FIG. 6.** Cell death genes are transcribed in dying larval midguts, and mutations in steroid-regulated genes impact transcription of cell death genes. Midguts were dissected from Canton S wild-type, and *BR-C* ($2Bc^2/Y$) and *E93* ($E93^1/Df(3R)93F^{x2}$) mutants, staged as midthird instar larvae (-18), late third instar larvae (-8, -4), or at different times following puparium formation, as indicated at the top. Total RNA was extracted from these midguts and analyzed by Northern blot hybridization. The blots were hybridized with probes to detect the cell death regulators *rpr, hid, ark, dronc,* and *crq*. Hybridization with *rp49* was used as a control for loading and transfer.

illustrate the complexity of developmental cell death and indicate the importance of studying this fundamental cellular process *in vivo*.

### *Larval Midgut Cell Death Is Independent of Adult Midgut Formation*

The dramatic reorganization of midgut structure during *Drosophila* metamorphosis involves the destruction of larval cells and the proliferation and morphogenesis of adult midgut cells (Robertson, 1936). A similar reorganization occurs during metamorphosis of other holometabolous insects that have been analyzed (Komuves *et al.*, 1985; Nopanitaya and Misch, 1974; Pipan and Rakovec, 1980; Priester, 1972), and is likely required because of the huge change in diet between the larval and adult stages. In *Drosophila*, larvae live in and eat decaying fruit, while mobile winged adults consume a semiliquid diet. In insects such as mosquitoes, larvae live in water and consume microorganisms, while the adult diet is nectar and blood. This reorganization of gut structure is not restricted to insects, as vertebrate organisms such as amphibians also exhibit dramatic changes in gut structure during metamorphosis (Shi and Ishizuya-Oka, 1996).

This study indicates that adult midgut epithelium formation precedes and is independent of larval midgut cell death. This is not only logical, but may be essential for survival. The digestive system represents an important barrier to pathogens (Basset *et al.*, 2000) and, thus, it may be critical to develop an adult midgut epithelium before the larval midgut structure is compromised. Although we did observe the early stages of autophagy in late third larval instar midguts immediately following the stage that the ecdysone titer rises (Fig. 4), the adult midgut epithelium was clearly formed by the stage larval midgut cell death was reaching advanced morphological stages (Figs. 1 and 4). Clearly, these cellular changes are extremely synchronized, and it will be interesting to determine the relationship of dying larval and differentiating adult cells and to test whether they develop autonomously in their response to ecdysone.

### *Steroid Regulation of Midgut Cell Death Occurs by Autophagy*

*Drosophila* larval midguts undergo rapid programmed cell death in response to the steroid ecdysone (Jiang *et al.*, 1997). Markers that are usually associated with apoptosis, including nuclear staining by acridine orange, TUNEL to

detect DNA fragmentation, and exposure of phosphatidylserine on the outer leaflet of the plasma membrane, are observed in dying larval midguts (Jiang *et al.*, 1997). However, *Drosophila* midguts die by autophagic programmed cell death (Fig. 4), as do the larval midguts of other holometabolous insects (Komuves *et al.*, 1985; Nopanitaya and Misch, 1974; Pipan and Rakovec, 1980; Priester, 1972). We have expanded on previous studies by investigating the cellular changes that occur during midgut cell death and by providing a molecular genetic mechanism for how *Drosophila* midgut cell death is regulated. Autophagy and apoptosis are the most prominent forms of programmed cell death that occur during animal development, and autophagy appears to occur when groups of cells or entire tissues die (Clarke, 1990; Schweichel and Merker, 1973). Numerous cells die when ecdysone triggers *Drosophila* midgut destruction, and, thus, it is not surprising that these cells die by autophagy. Furthermore, the larval midgut does not show advanced signs of autophagy until it has been isolated from the haemocoel by the developing adult midgut epithelium (Figs. 1 and 4). Therefore, professional phagocytes of the blood cell lineage have no direct access to the dying larval midgut, and these larval midgut cells must degrade themselves, fulfilling the definition of autophagy.

Morphological studies of dying cells suggest that apoptosis and autophagy are regulated by distinct mechanisms. In contrast, studies of both salivary glands and midguts in *Drosophila* indicate that these two forms of cell death share some common biochemical properties. Expression of the caspase inhibitor p35 blocks both salivary gland and midgut programmed cell death (Jiang *et al.*, 1997). Furthermore, the core cell death genes *rpr*, *hid*, *ark*, and *dronc* and the CD36 relative *crq*, a gene that is required for removal of apoptotic cells during *Drosophila* embryogenesis, exhibit increased transcription during salivary gland and midgut autophagy (Lee *et al.*, 2000). The clear differences in the morphology of apoptotic and autophagic cells indicate that differences exist in the mechanisms of their destruction. For example, apoptotic cells require phagocytes to complete lysosomal degradation, whereas autophagic cells appear capable of completing degeneration without the assistance of other cells. Future studies of the similarities and differences between apoptosis and autophagy will have important implications in understanding the mechanisms that underlie these most prominent forms of developmental cell death.

### *Differences in Dying Midguts and Salivary Glands Reflect Distinct Cell Death Mechanisms*

Studies of ecdysone-triggered destruction of *Drosophila* larval midguts and salivary glands illustrate many similarities in these dying cells (Jiang *et al.*, 1997; Lee and Baehrecke, 2001). However, several important differences exist between ecdysone-regulated midgut and salivary gland programmed cell death. First, these two tissues are triggered to die by independent pulses of ecdysone (Jiang *et al.*, 1997). While the nuclear receptor βFTZ-F1 is responsible for specifying ecdysone induction of *BR-C*, *E74A*, and *E93* immediately prior to larval salivary gland programmed cell death (Broadus *et al.*, 1999; Woodard *et al.*, 1994), the factor(s) that specify the timing of the cell death response in larval midguts 12 h earlier remain unclear. *BR-C* and *E93* appear to be critical regulators of midgut cell death (Figs. 2, 5, and 6), but it is unclear how the ecdysone receptor complex activates these genes in midguts. βFTZ-F1 is not expressed in midguts prior to ecdysone-induced cell death of this tissue (E.H.B., unpublished observations), so other factors must be responsible for induction of *BR-C* and *E93* in midguts. One possibility is that the hormone receptor complex activates *BR-C* and *E93* independently of a factor such as βFTZ-F1. Alternatively, another nuclear receptor, or possibly an unrelated transcription regulator, may regulate *BR-C* and *E93*. Future genetic studies and analyses of the *BR-C* and *E93* promoters will define the mechanism for the stage-specific induction of cell death by ecdysone in larval midguts.

The distributed association of future adult cells within the epithelium of larval midguts (Robertson, 1936) is another important difference between ecdysone-regulated midgut and salivary gland programmed cell death. The close association of larval and adult midgut cells may be one of the reasons why larval midgut exhibits a less synchronized cell death than salivary glands. Both salivary glands and midguts require the function of the *E93* and *BR-C* genes. However, mutations in these genes appear to result in different effects in salivary glands and midguts; *BR-C* appears to play a more important role in midguts. While both salivary glands and midguts express the cell death genes *rpr*, *hid*, *ark*, *dronc*, and *crq*, the impact of mutations in *BR-C* and *E93* are very different in the midgut than in salivary glands. *BR-C* affects transcription of *rpr*, *hid*, and *crq*, but *E93* mutants only affect *dronc* transcription in midguts (Fig. 6). In contrast, mutations in *E93* prevent proper transcription of all of these cell death genes in dying salivary glands (Lee *et al.*, 2000). Clearly, many more genes may be involved in the complicated autophagic cell death of midguts. While we have identified several similarities and differences between salivary gland and midgut death, future analyses are needed to clarify the mechanism by which the steroid ecdysone triggers midgut programmed cell death.

## ACKNOWLEDGMENTS

We thank T. Maugel, L. von Kalm, C. Bayer, M. Muskavitch, M. Dushay, the Laboratory of Biological Ultrastructure, and the Bloomington *Drosophila* Stock Center for reagents, flies, and helpful discussions. This work was supported by NIH Grant GM59136 (to E.H.B.).

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Received for publication April 25, 2002
Revised July 5, 2002
Accepted July 5, 2002
Published online August 26, 2002