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A Steroid-Triggered Transcriptional Hierarchy Controls Salivary Gland Cell Death during *Drosophila* Metamorphosis

Changan Jiang,* Anne-Françoise J. Lamblin,†§ Hermann Steller,† and Carl S. Thummel*‡
*Howard Hughes Medical Institute
Department of Human Genetics
University of Utah
Salt Lake City, Utah 84112
†Howard Hughes Medical Institute
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

The steroid hormone ecdysone signals the stage-specific programmed cell death of the larval salivary glands during *Drosophila* metamorphosis. This response is preceded by an ecdysone-triggered switch in gene expression in which the *diap2* death inhibitor is repressed and the *reaper* (*rpr*) and *head involution defective* (*hid*) death activators are induced. Here we show that *rpr* is induced directly by the ecdysone–receptor complex through an essential response element in the *rpr* promoter. The *Broad-Complex* (*BR-C*) is required for both *rpr* and *hid* transcription, while *E74A* is required for maximal levels of *hid* induction. *diap2* induction is dependent on βFTZ-F1, while *E75A* and *E75B* are each sufficient to repress *diap2*. This study identifies transcriptional regulators of programmed cell death in *Drosophila* and provides a direct link between a steroid signal and a programmed cell death response.

Introduction

Programmed cell death provides a critical means by which multicellular organisms remove obsolete or damaged cells. Unlike necrosis, which results from external injury, apoptosis is initiated inside the cell through an evolutionarily conserved genetic regulatory pathway and is characterized by distinct morphological and biochemical changes (Kerr et al., 1972; Jacobson et al., 1997; Vaux and Korsmeyer, 1999). Over the past few years, significant advances have been made in our understanding of the cell death pathway and how it is regulated by intracellular and extracellular signals. Though the cell death machinery appears to be present in all cells, its activation is determined by a balance between death activators and death inhibitors. Multiple signals, including growth factors, DNA-damaging agents, and hormones, can influence the balance between these regulators and thereby induce or suppress cell death in a precisely regulated manner. This level of control is critical to the survival of the animal, as misregulation of programmed cell death has been associated with a wide range of human diseases (Thompson, 1995; Rudin and Thompson, 1997).

Hormones play a key role in controlling apoptosis. The term "programmed cell death" was first used by Lockshin and Williams (1965) in their study of steroid-regulated intersegmental muscle degeneration during silkmoth metamorphosis. Similarly, thyroid hormone–induced RNA and protein synthesis are required for the destruction of the tadpole tail during *Xenopus* metamorphosis (Tata, 1966). Steroid hormones also control programmed cell death in a wide range of mammalian tissues, including neurons, mammary glands, testes, the prostate, ovaries, and the uterus (Kiess and Gallaher, 1998). The best studied vertebrate model for hormone-regulated programmed cell death is the apoptosis of immature thymocytes and mature T cells in response to glucocorticoids. This response is dependent on the steroid, the glucocorticoid receptor, and de novo transcription, suggesting that the hormone is triggering a death-specific genetic program (Evans-Storms and Cidlowski, 1995). The genes that direct this death response, however, remain unknown.

The programmed cell death of larval tissues during *Drosophila* metamorphosis provides an ideal system to define the molecular mechanisms of steroid-regulated apoptosis. Metamorphosis in *Drosophila* is controlled by pulses of the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone) (Riddiford, 1993). A pulse of ecdysone at the end of larval development triggers puparium formation, signaling the onset of metamorphosis and initiating the prepupal stage in development. This is followed by another ecdysone pulse, approximately 12 hr after puparium formation, that signals the prepupal–pupal transition. In response to these sequential ecdysone pulses, obsolete larval tissues are destroyed in a stage-specific manner as adult tissues and structures develop from small clusters of progenitor cells, resulting in the transformation of a larva into an immature fly (Robertson, 1936). The larval midgut initiates cell death at puparium formation in response to the late larval pulse of ecdysone, while the salivary glands are not destroyed until approximately 15 hr after puparium formation, in response to the prepupal pulse of ecdysone. The destruction of these tissues is accompanied by nuclear staining with acridine orange and DNA fragmentation, indicative of apoptosis (Jiang et al., 1997). Furthermore, midgut and salivary gland cell death can be inhibited by ectopic expression of the p35 anti-apoptotic protein, implicating a role for caspases in the death response. This is consistent with ecdysone-induction of the DRONC caspase in both the larval midgut and salivary glands before the onset of cell death (Dorstyn et al., 1999). The larval midgut and salivary glands thus appear to be destroyed by a steroid-triggered programmed cell death pathway that has hallmark features of apoptosis.

As in vertebrate organisms, caspase activation in *Drosophila* is controlled by both cell death activators and

‡To whom correspondence should be addressed (e-mail: carl.thummel@genetics.utah.edu).
§Present address: National Cancer Institute, National Institutes of Health, Building 37, Room 4D11, 37 Convent Drive, Bethesda, Maryland 20892.

cell death inhibitors (Bergmann et al., 1998b; Abrams, 1999). Three cell death activators have been identified in *Drosophila*—*reaper* (*rpr*), *head involution defective* (*hid*), and *grim* (White et al., 1994; Grether et al., 1995; Chen et al., 1996). Removal of these genes by use of the *Df(3L)H99* deletion effectively blocks programmed cell death in *Drosophila* (White et al., 1994). In addition, specific *hid* alleles lead to embryonic lethality with defects in programmed cell death (Grether et al., 1995). The expression patterns of *rpr* and *grim* foreshadow cell death during *Drosophila* development, whereas *hid* is also expressed in some cells that fail to die (White et al., 1994; Grether et al., 1995; Chen et al., 1996; Robinow et al., 1997). Ectopic expression of each of these genes is sufficient to induce cell death by activating a caspase cascade. More recent studies, however, indicate that *rpr* and *hid*, or *rpr* and *grim*, function together in a cooperative manner to direct death in some cell types (Zhou et al., 1997; Draizen et al., 1999). Cell death in *Drosophila* can also be triggered by UV or X irradiation, as in mammalian cells, and this process is preceded by induction of *rpr* transcription (Nordstrom et al., 1996; A.-F. Lamblin and H. Steller, submitted). Taken together, these observations indicate that expression of the *Drosophila* death activators serves as a central life/death switch in response to different stimuli. It seems likely that these genes are controlled by *cis*-acting regulatory elements that function together to integrate the input from a number of cell death signaling pathways, precisely coordinating the patterns of cell death during development. Hence, understanding the regulation of *rpr*, *hid*, and *grim* transcription provides a critical step for defining the control of programmed cell death in *Drosophila*.

Like vertebrate cells, the fate of a *Drosophila* cell is also modulated by IAP homologs (Inhibitor of Apoptosis proteins) that function as cell death inhibitors (Crook et al., 1993; Birnbaum et al., 1994; Clem and Miller, 1994). Two IAP-encoding genes have been described in *Drosophila*, *diap1* and *diap2* (Hay et al., 1995; Duckett et al., 1996). Ectopic expression of *diap1* or *diap2* is sufficient to block cell death driven by *rpr* or *hid* in the *Drosophila* eye. In addition, *diap1* null mutants die during embryogenesis with extensive cell death, indicating that this gene is essential for inhibiting the death response during embryonic development (Wang et al., 1999; Goyal et al., 2000). These studies have demonstrated that *diap1* blocks caspase activation and that this inhibition is relieved through interactions with RPR, HID, or GRIM. This is consistent with the ability of *Drosophila* IAPs to interact physically with RPR, HID, and GRIM (Vucic et al., 1997, 1998) as well as the observation that DIAP1 can directly inhibit DCP-1 caspase activity (Hawkins et al., 1999).

Both *rpr* and *hid* appear to play a key role in the stage-specific destruction of the larval midgut and salivary glands during metamorphosis (Jiang et al., 1997). These genes are coordinately induced in the midguts of newly formed prepupae, as this tissue enters programmed cell death. In contrast, *rpr* and *hid* are not induced in the salivary glands until 12–14 hr after puparium formation, several hours before the glands are destroyed. Interestingly, the induction of *rpr* and *hid* in the salivary glands is preceded by a brief burst of *diap2* expression. This switch in death gene expression suggests that ecdysone triggers salivary gland cell death by simulaneously repressing a death inhibitor and inducing death activators, shifting the balance between pro- and antiapoptotic activities.

Ecdysone functions through a heterodimeric receptor comprised of two members of the nuclear receptor superfamily, EcR (NR1H1) and the RXR homolog, Ultraspiracle (USP, NR2B4) (Koelle et al., 1991; Koelle, 1992; Yao et al., 1992; Thomas et al., 1993). The ecdysone–EcR/USP complex directly regulates primary response genes, including three early genes that were originally identified as ecdysone-inducible puffs in the larval salivary gland polytene chromosomes—the *Broad-Complex* (*BR-C*), *E74*, and *E75* (Russell and Ashburner, 1996). The *BR-C* encodes a family of zinc finger transcription factors (DiBello et al., 1991). *E74* encodes two isoforms of an ETS domain transcription factor, designated E74A and E74B (Burtis et al., 1990), and *E75* (NR1D3) encodes three orphan members of the nuclear receptor superfamily, designated E75A, E75B, and E75C (Segraves and Hogness, 1990). These early genes, in turn, regulate large batteries of secondary response late genes, defining ecdysone-triggered regulatory hierarchies (Russell and Ashburner, 1996; Thummel, 1996). Genetic studies have demonstrated that the *BR-C* and *E74* are required for appropriate developmental responses to ecdysone during metamorphosis (Kiss et al., 1988; Restifo and White, 1992; Fletcher et al., 1995). Furthermore, BR-C and E74A proteins have been shown to directly regulate late gene transcription (Urness and Thummel, 1995; Crossgrove et al., 1996). Ectopic expression studies have demonstrated that E75B can repress the βFTZ-F1 orphan nuclear receptor (NR5A3), suggesting that it contributes to the timing of β*FTZ-F1* expression in midprepupae (White et al., 1997). Loss-of-function and gain-of-function genetic studies have indicated that βFTZ-F1 functions as a competence factor to facilitate reinduction of early gene transcription in response to the prepupal pulse of ecdysone (Woodard et al., 1994; Broadus et al., 1999).

In this paper, we integrate *diap2*, *rpr*, and *hid* regulation into the ecdysone-triggered hierarchies that control salivary gland cell death. We show that β*FTZ-F1* is required for the induction of *diap2* in late prepupal salivary glands and that *E75A* or *E75B* is sufficient to repress this death inhibitor, defining the brief burst of *diap2* expression that precedes *rpr* and *hid* induction. Organ culture experiments reveal that *rpr* is induced as both a primary and secondary response to ecdysone, while *hid* is induced solely as a secondary response to the hormone, consistent with the timing of their induction in staged salivary glands. The EcR/USP heterodimer directly regulates *rpr* transcription through an essential binding site in its promoter. The *BR-C* is required for both *rpr* and *hid* induction, and *E74A* is required for maximal *hid* transcription, indicating that ecdysone-induced transcription factors function together with the ecdysone receptor to induce cell death activators. This study defines the ecdysone-triggered genetic cascades that precede the destruction of the larval salivary glands providing a foundation for understanding how steroid hormones might regulate programmed cell death in other higher organisms.

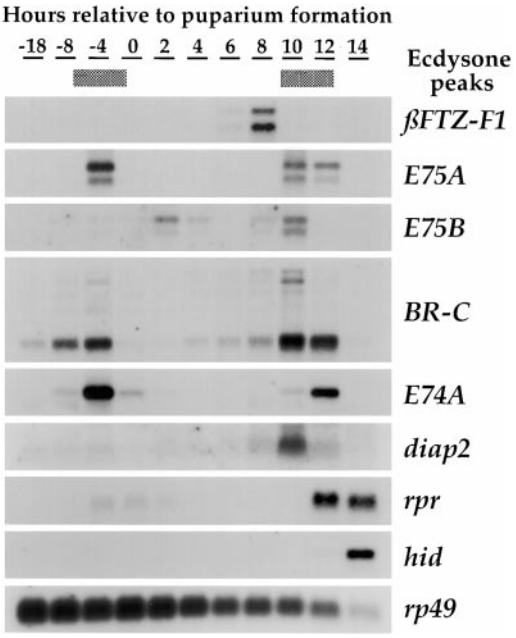

Figure 1. Temporal Profiles of Ecdysone-Regulated Gene Expression in Staged Salivary Glands

Total RNA was isolated from 12 pairs of salivary glands dissected from staged Canton-S third instar larvae, prepupae, or pupae, and analyzed by Northern blot hybridization. Probes were used to detect β*FTZ-F1*, *BR-C*, *E74A*, *E75A*, *E75B*, *diap2*, *rpr*, and *hid* mRNAs. The timing of the late larval and prepupal ecdysone peaks is shown at the top (Riddiford, 1993). *diap1* mRNA cannot be detected in salivary glands at these stages of development (data not shown), and *EcR* and *usp* are expressed throughout this time course (Koelle et al., 1991; Andres et al., 1993; Henrich et al., 1994). Hybridization to detect *rp49* mRNA was used as a control for loading and transfer. The level of *rp49* mRNA declines gradually in the larval salivary glands during prepupal and pupal development (Baehrecke and Thummel, 1995).

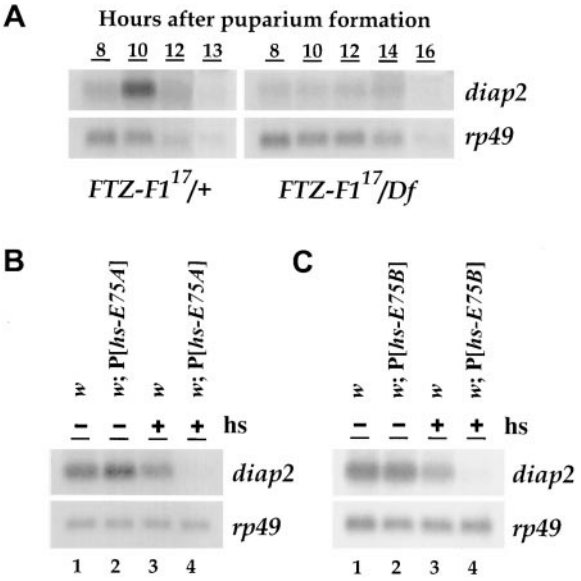

Figure 2. β*FTZ-F1* Is Required for *diap2* Induction, and *E75A* or *E75B* Is Sufficient to Repress *diap2* Transcription

(A) Total RNA was extracted from 12 pairs of salivary glands dissected from staged *FTZ-F1*^{17}/+ (control) and *FTZ-F1*^{17}/*Df(3L)Cat*^{DH104} prepupae and pupae, and the expression of *diap2* was determined by Northern blot hybridization. *rp49* mRNA was used as a control for loading and transfer.

(B) Both *w*^{1118} (lane 3) or *w*^{1118}, P[*hs-E75A*] (lane 4) 7.5 hr prepupae were incubated at 37°C for 30 min and allowed to recover at 25°C for 2 hr. RNA was extracted from the salivary glands and analyzed by Northern blot hybridization. As a control, RNA was also isolated from the salivary glands of 10 hr *w*^{1118} (lane 1) and *w*^{1118}, P[*hs-E75A*] (lane 2) prepupae that had not been subjected to heat treatment. Probes were used to detect *diap2* transcription as well as *rp49* mRNA as a control for loading and transfer.

(C) A similar experiment as in (B) was performed to study the effect of ectopic *E75B* expression on *diap2* transcription.

Results

β*FTZ-F1* and *E75* Determine the Duration of *diap2* Expression

β*FTZ-F1* expression immediately precedes that of *diap2* in larval salivary glands (Figure 1) and is required for ecdysone-induced gene expression in late prepupae (Broadus et al., 1999). These observations led to the hypothesis that β*FTZ-F1* may induce *diap2* expression. To test this possibility, we examined *diap2* transcription in the salivary glands of *FTZ-F1*^{17} mutant prepupae. *FTZ-F1*^{17} is a hypomorphic β*FTZ-F1* allele that leads to severe defects in both genetic and biological responses to the prepupal pulse of ecdysone (Broadus et al., 1999). Salivary glands were dissected from staged *FTZ-F1*^{17}/+ controls and *FTZ-F1*^{17}/*Df(3L)Cat*^{DH104} mutant prepupae, and *diap2* transcription was examined by Northern blot hybridization. The levels of *diap2* mRNA are significantly reduced in β*FTZ-F1* mutant salivary glands, indicating that *diap2* expression is dependent on β*FTZ-F1* function (Figure 2A).

Since *diap2* transcription is repressed by ecdysone in cultured larval salivary glands, it seems likely that one or more ecdysone-induced transcription factors may mediate this response (Jiang et al., 1997). Two lines of evidence suggest that *E75A* and/or *E75B* could encode this repressive function. First, E75A and E75B can function as repressors (White et al., 1997; W. Segraves, personal communication), much like their vertebrate homolog, the Rev-Erb orphan nuclear receptor (NR1D1) (Harding and Lazar, 1995; Adelmant et al., 1996). Second, both *E75A* and *E75B* are induced by ecdysone immediately before the decline in *diap2* transcription (Figure 1). To test this model, we ectopically expressed *E75A* or *E75B* in late prepupal salivary glands, when *diap2* is maximally transcribed. Control, P[*hs-E75A*], and P[*hs-E75B*] 7.5 hr prepupae were maintained at either 25°C or 37°C for 30 min and allowed to recover for 2 hr at room temperature. Salivary glands were dissected from these approximately 10 hr prepupae, and the level of *diap2* transcription was determined by Northern blot hybridization. As expected, high levels of *diap2* mRNA can be detected in salivary glands that had not been subjected to heat treatment (Figures 2B and 2C, lanes 1 and 2). Heat treatment slightly reduced *diap2* expression in the salivary glands of control prepupae, most likely due to the slight developmental delay associated with heat treatment (Figures 2B and 2C, lane 3). Strikingly, however, *diap2* mRNA was undetectable in the salivary glands of heat-treated P[*hs-E75A*] and P[*hs-E75B*] transgenic animals, indicating that either *E75A* or

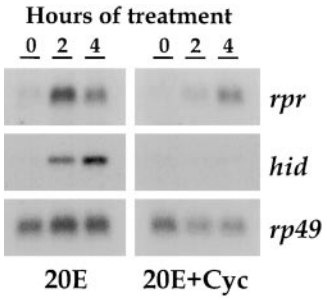

Figure 3. *rpr* and *hid* Transcription Is Induced by Ecdysone in Cultured Larval Salivary Glands

Salivary glands were dissected from 10 hr prepupae and cultured in the presence of either 20-hydroxyecdysone alone (20E) or 20-hydroxyecdysone and cycloheximide (20E + Cyc). Total RNA was isolated after 0, 2, or 4 hr of culture, and *rpr* and *hid* mRNA was detected by Northern blot hybridization. *rp49* mRNA was detected as a control for loading and transfer.

E75B is sufficient to repress *diap2* transcription (Figures 2B and 2C, lane 4). These observations support the hypothesis that *E75* normally represses *diap2* transcription in late prepupal salivary glands.

Both *rpr* and *hid* Are Induced by Ecdysone in Late Prepupal Salivary Glands

Although our initial studies indicated that *rpr* and *hid* are coordinately induced in the salivary glands approximately 12 hr after puparium formation (Jiang et al., 1997), more recent work has shown that *rpr* is induced approximately 1.5 hr earlier than *hid*, suggesting that these death activators are regulated by distinct mechanisms (Figure 1 and data not shown). The timing of *rpr* induction is synchronous with the prepupal ecdysone pulse, suggesting that it may be induced as a primary response to the hormone, while the delay in *hid* induction suggests that it may be a secondary response to ecdysone (Figure 1).

These two modes of regulation can be distinguished by their different sensitivity to the inhibition of protein synthesis (Ashburner, 1974). Salivary glands were dissected from 10 hr wild-type prepupae and cultured in insect medium supplemented with 20-hydroxyecdysone, either in the presence or absence of cycloheximide. Total RNA was extracted after 0, 2, or 4 hr of culture and analyzed by Northern blot hybridization (Figure 3). Both *rpr* and *hid* are induced within 2 hr of hormone treatment, consistent with the proposal that these genes are induced by ecdysone in late prepupal salivary glands. In the presence of the protein synthesis inhibitor cycloheximide, *rpr* transcription is both delayed and reduced, while *hid* expression is completely eliminated (Figure 3). These observations indicate that *rpr* is induced directly by the hormone–receptor complex, although maximal levels of *rpr* transcription also require the synthesis of ecdysone-induced proteins. In contrast, *hid* is induced solely as a secondary response to ecdysone. These observations are consistent with the timing of *rpr* and *hid* induction in staged salivary glands (Figure 1) and provide a framework for defining the molecular mechanisms by which ecdysone regulates *rpr* and *hid* transcription, triggering salivary gland cell death.

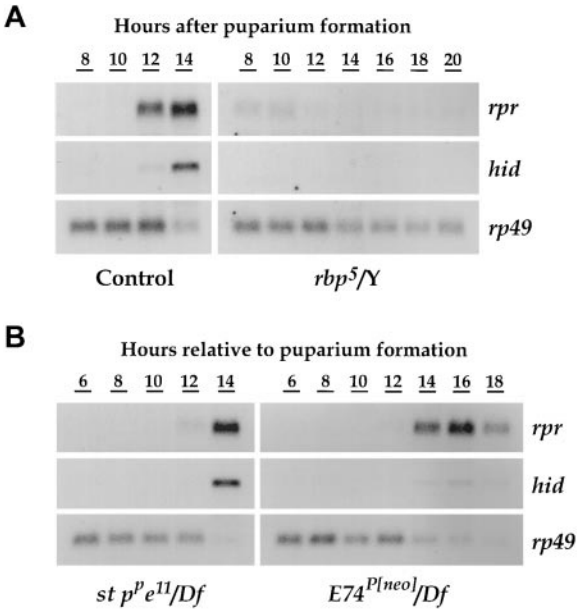

Figure 4. The *BR-C* and *E74A* Regulate *rpr* and *hid* Transcription

(A) Salivary glands were dissected from staged control (*Binsn*/Y or *y rbp*^{5}*/Binsn*) and *rbp* mutant (*y rbp*^{5}/Y) prepupae and pupae. RNA was isolated and analyzed by Northern blot hybridization for the patterns of *rpr* and *hid* transcription.

(B) Salivary glands were dissected from staged control [*st p*^{p} *e*^{11}/*Df(3L)st-81k19*] and *E74A* mutant [*E74*$^{P[neo]}$*/Df(3L)st-81k19*] prepupae and pupae. RNA was isolated and analyzed by Northern blot hybridization for the patterns of *rpr* and *hid* transcription. There is a slight delay in the prepupal ecdysone pulse in the *Df(3L)st-81k19* genetic background. *rp49* mRNA was detected as a control for loading and transfer. The level of *rp49* mRNA declines gradually during prepupal and pupal development (Baehrecke and Thummel, 1995).

The *BR-C* Is Required for Both *rpr* and *hid* Induction

The *BR-C* is defined by three genetic functions: *broad* (*br*), *reduced bristles on palpus* (*rbp*), and *l(1)2Bc* (Belyaeva et al., 1980, 1982; Kiss et al., 1988). Earlier studies have shown that the *rbp* function of the *BR-C* is required for salivary gland cell death during metamorphosis (Restifo and White, 1992; Zhimulev et al., 1995). We have confirmed this result by finding that larval salivary glands are not destroyed by 22 hr after puparium formation in pupae that carry the *rbp*^{5} null allele (n = 20; data not shown). The high penetrance of this mutant phenotype suggests that *rpr* and *hid* may not be properly expressed in *rbp*^{5} mutant salivary glands.

To test this hypothesis, salivary glands were dissected from staged *rbp*^{5} mutants, and *rpr* and *hid* expression was examined by Northern blot hybridization. Both *rpr* and *hid* transcription is significantly reduced in *rbp*^{5} mutant salivary glands, indicating that the failure of salivary gland cell death in this mutant can be attributed to its inability to express these death activators (Figure 4A). Both β*FTZ-F1* and the ecdysone-inducible *E93* early gene are expressed in *rbp*^{5} mutant salivary glands, indicating that the block in *rpr* and *hid* transcription is not simply due to developmental arrest of the mutant animals (data not shown). It should be noted that the *BR-C* is expressed in midprepupal salivary glands and thus would be present in the late prepupal glands used for

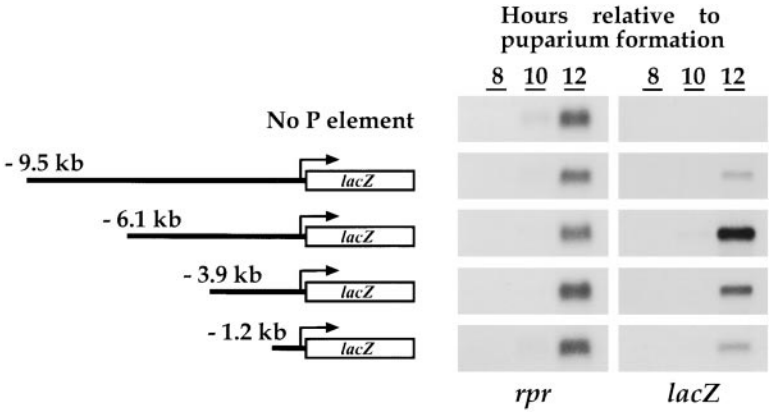

Figure 5. A 1.3 kb Region of the *rpr* Promoter Is Sufficient to Direct Proper Temporal Regulation in Larval Salivary Glands

Transgenic lines were established that carry either 9.5, 6.1, 3.9, or 1.2 kb of *rpr* genomic sequences located upstream from the start site of transcription, fused to a *lacZ* reporter gene. Salivary glands were dissected from these transgenic animals, as well as a $y\ w^{67}$ control, at either 8, 10, or 12 hr after puparium formation. *lacZ* transcription was detected by Northern blot hybridization and compared with that of the endogenous *rpr* gene. Approximately equal amounts of RNA were loaded in each lane, and this was confirmed by hybridization with an *rp49* probe (data not shown). Representative results are depicted from analyses of two to three independent transformant lines carrying each P element construct.

the cycloheximide experiment described above (Figure 1; Emery et al., 1994). This explains why the reduced level of *rpr* transcription observed in the absence of protein synthesis (Figure 3) is not as severe as the rbp^5 mutant phenotype (Figure 4A).

E74A Is Required for Maximal *hid* Induction

Both molecular and genetic studies have indicated that the *BR-C* and *E74* function together in common developmental pathways during the onset of metamorphosis (Fletcher and Thummel, 1995; Urness and Thummel, 1995; Crossgrove et al., 1996). This led us to ask whether, like the *BR-C*, *E74* might contribute to the ecdysone-triggered destruction of larval salivary glands. In support of this proposal, salivary gland cell death is significantly delayed in $E74^{P[neo]}$ animals. This mutation is a null allele that inactivates the *E74A* promoter (Fletcher et al., 1995). While salivary glands in control animals are completely destroyed by 16 hr after puparium formation, approximately 20% of $E74^{P[neo]}Df(3L)st\text{-}81k19$ animals have salivary glands at 24 hr after puparium formation ($n = 30$; data not shown). This partially penetrant cell death defect suggests that *rpr* and *hid* expression may be reduced in *E74A* mutant salivary glands.

To test this hypothesis, salivary glands were dissected from staged $E74^{P[neo]}/Df(3L)st\text{-}81k19$ mutants, and *rpr* and *hid* expression was examined by Northern blot hybridization. Although *rpr* transcription is unaffected by the $E74^{P[neo]}$ mutation, the levels of *hid* transcription are significantly reduced (Figure 4B). This observation indicates that *E74A* is required for the maximal induction of *hid* but not *rpr*.

A 1.3 kb Region of the *rpr* Promoter Is Sufficient to Direct Proper Temporal Regulation of *rpr* Transcription in Doomed Salivary Glands

The observation that *rpr* transcription is induced directly by ecdysone in cultured larval salivary glands (Figure 3) indicates that one or more EcR/USP binding sites should be present in the *rpr* promoter. As a first step toward identifying these regulatory elements, we mapped the sequences required for ecdysone-inducible *rpr* transcription in larval salivary glands. An earlier study has shown that 9.6 kb of the *rpr* promoter is sufficient to recapitulate certain aspects of the complex pattern of *rpr* expression during embryogenesis (A.-F. Lamblin and H. Steller, submitted). Four P element constructs were made that carry either 9.5, 6.1, 3.9, or 1.2 kb of DNA upstream from the *rpr* transcription start site and 125 bp downstream from the transcription start site, with the *rpr* 5′ untranslated region fused to a *lacZ* reporter gene. These constructs were introduced into the *Drosophila* germline by P element–mediated transformation, and the patterns of *lacZ* transcription in staged salivary glands were compared with those of the endogenous *rpr* gene by Northern blot hybridization (Figure 5). An increased level of *rpr* promoter activity is seen upon deletion of sequences between −9.5 and −6.1 kb relative to the start site of *rpr* transcription. The overall level of *lacZ* transcription is then reduced as more *rpr* regulatory sequences are deleted. However, 1.3 kb of the *rpr* promoter is sufficient to direct *lacZ* induction in synchrony with that of the endogenous *rpr* gene, indicating that this region contains the sequences required for proper temporal regulation.

The Ecdysone Receptor Directly Regulates *rpr* Transcription

A mobility shift competition assay was used to identify EcR/USP binding sites within the 1.3 kb *rpr* promoter fragment. An oligonucleotide containing the strong EcR/USP binding site from the *hsp27* gene, the *hsp27* EcRE, was used as a probe for this assay (Riddihough and Pelham, 1987). The *hsp27* EcRE can be effectively bound by the EcR/USP heterodimer in a mobility shift assay (Figure 6B, lanes 4–6). DNA fragments from the 1.3 kb *rpr* promoter region were generated by PCR and tested for their ability to compete with labeled *hsp27* EcRE for binding by the EcR/USP complex. A 274 bp fragment extending from −195 bp to +80 bp relative to the *rpr* transcription start site significantly reduced the binding of EcR/USP to the *hsp27* EcRE, while six other fragments from the 1.3 kb region did not (data not shown). Sequence analysis revealed a single imperfect palindromic EcR/USP binding site within this fragment. This *rpr* EcRE matches 10 out of 13 positions with the consensus EcR/USP binding site defined by Lehmann and Korge (1995)

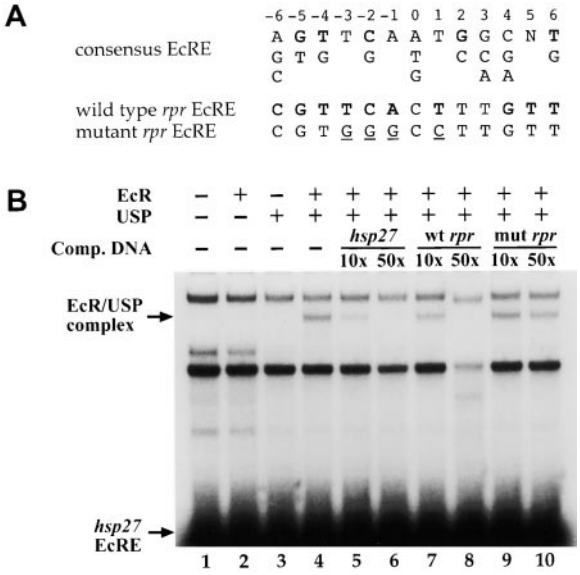

Figure 6. The *rpr* Promoter Contains an EcR/USP Binding Site

(A) Shown at the top is a consensus sequence derived from the EcREs of four genes: *hsp23*, *hsp27*, *Eip28/29*, and *Fbp1* (Lehmann and Korge, 1995). Bold letters indicate the preferred nucleotide in cases where more than one nucleotide can be used. Below is shown the wild-type *rpr* EcRE, with bold letters marking nucleotides that match the consensus sequence. At the bottom is the sequence of the mutated *rpr* EcRE in which conserved nucleotides, T(−3), C(−2), A(−1), and T(+1), were changed to G or C (underlined).

(B) ^{32}P-labeled *hsp27* EcRE was incubated with either reticulocyte lysate, in vitro translated EcR protein, in vitro translated USP protein, or both EcR and USP. The DNA–protein complexes were fractionated by electrophoresis and visualized by autoradiography. Addition of EcR and USP resulted in the formation of a specific EcR/USP/*hsp27* EcRE complex (upper arrow) resolved from the unbound *hsp27* EcRE (lower arrow). Addition of a 10-fold molar excess of unlabelled *hsp27* EcRE significantly reduced the binding of EcR/USP to the labeled *hsp27* EcRE (lane 5). A 10-fold molar excess of the wild-type *rpr* EcRE also competed for binding, although not as effectively as the *hsp27* EcRE (lane 7), while a 10-fold molar excess of the mutant *rpr* EcRE did not significantly compete for binding (lane 9).

(Figure 6A). Moreover, an oligonucleotide carrying this sequence can be bound by the EcR/USP heterodimer in vitro (data not shown). Addition of a 10-fold molar excess of unlabeled *rpr* EcRE resulted in an approximately 40% decrease in the ability of EcR/USP to bind to the *hsp27* EcRE, indicating that the *rpr* element is not as strong of a binding site as the canonical *hsp27* element (Figure 6B, lane 7). This observation is consistent with the deviations from the consensus at positions +2 and +3 in the *rpr* EcRE (Figure 6A). An oligonucleotide was synthesized in which four highly conserved nucleotides in the *rpr* EcRE were altered, at positions −3, −2, −1, and +1 (Figure 6A). A 10-fold molar excess of this mutated EcRE had no effect on formation of EcR/USP/*hsp27* EcRE complex, indicating that these conserved nucleotides are required for binding of the *rpr* EcRE by the EcR/USP heterodimer (Figure 6B, lane 9).

Site-directed mutagenesis was used to replace the EcRE within the 1.3 kb *rpr* promoter with the mutated *rpr* EcRE sequence shown in Figure 6A. The mutant *rpr* promoter fragment was then fused with a *lacZ* reporter gene and introduced into the genome by P element–

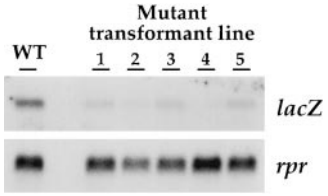

Figure 7. The *rpr* EcRE Is Required for Transcription

Site-directed mutagenesis was used to change four nucleotides in the EcRE of the 1.3 kb *rpr* promoter fragment, creating the mutant *rpr* EcRE shown in Figure 6A. The mutant 1.3 kb *rpr* promoter was fused with a *lacZ* reporter gene and introduced into the genome by P element–mediated germline transformation. Total RNA was isolated from the salivary glands of transgenic animals carrying either the wild-type or mutant 1.3 kb *rpr* promoter at 12 hr after puparium formation, and *lacZ* and *rpr* mRNA was detected by Northern blot hybridization.

mediated transformation. Salivary glands were dissected from both the control wild-type 1.3 kb *rpr-lacZ* transformant line as well as five different mutant *rpr-lacZ* transformant lines at 12 hr after puparium formation. The levels of both *lacZ* and endogenous *rpr* transcription were determined by Northern blot hybridization. In each line carrying the mutant *rpr* EcRE, *lacZ* transcription was reduced to less than 10% of the level in the control line, indicating that the EcRE is required for *rpr* transcription in larval salivary glands (Figure 7). This result strongly suggests that the ecdysone–receptor complex directly regulates *rpr* transcription through at least one binding site in the *rpr* promoter.

Discussion

Steroid hormones act as key regulators of programmed cell death in higher organisms (Evans-Storms and Cidlowski, 1995; Kiess and Gallaher, 1998). In spite of this important function, little is known about the mechanisms by which a steroid signal is transduced to direct a programmed cell death response. Indeed, the link between specific signaling pathways and the induction of apoptosis represents a major challenge in understanding the regulation of cell death. In this study, we define a steroid-triggered genetic cascade that directs the programmed cell death of the larval salivary glands during *Drosophila* metamorphosis. The ecdysone-regulated transcription factors encoded by β*FTZ-F1*, *BR-C*, *E74*, and *E75* function together to direct a burst of the *diap2* death inhibitor followed by induction of the *rpr* and *hid* death activators. We propose that cooperation between *rpr* and *hid* allows them to overcome the inhibitory effect of *diap2*, precisely coordinating when the salivary glands are destroyed. We also provide evidence that the ecdysone–receptor complex directly induces *rpr* transcription through an essential response element in the promoter, providing a direct link between the steroid signal and a programmed cell death response.

β*FTZ-F1* and *E75* Define a Window of *diap2* Expression that Precedes Salivary Gland Cell Death

The *diap2* death inhibitor is expressed briefly in the salivary glands of late prepupae, foreshadowing the imminent destruction of this tissue. This transient expression is directed by at least three ecdysone-regulated

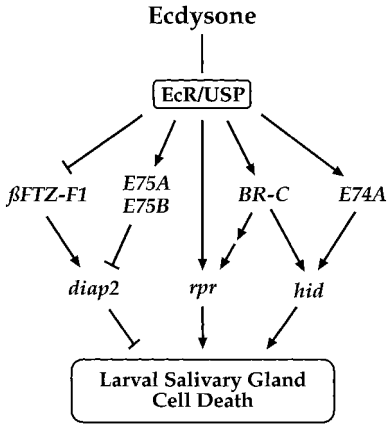

Figure 8. A Model for the Steroid Regulation of Salivary Gland Programmed Cell Death

β*FTZ-F1* induces the *diap2* death inhibitor in the salivary glands of late prepupae. The prepupal pulse of ecdysone then represses β*FTZ-F1* and induces *E75A* and *E75B*, which function in a redundant manner to repress *diap2*. The prepupal ecdysone pulse also directly induces *rpr* transcription as well as the transcription factors encoded by the *BR-C* and *E74A*. The *BR-C* is required for both *rpr* and *hid* transcription, and *E74A* is required for maximal levels of *hid* expression. Preliminary experiments indicate that *rpr* is indirectly regulated by the *BR-C*. See text for more details.

transcription factors—βFTZ-F1, E75A, and E75B (Figure 8). *diap2* induction is dependent on the βFTZ-F1 orphan nuclear receptor (Figure 2A). This is consistent with the timing of β*FTZ-F1* expression, which immediately precedes that of *diap2* (Figure 1), as well as the known role of β*FTZ-F1* as an activator of gene expression in late prepupae (Broadus et al., 1999).

The prepupal pulse of ecdysone represses β*FTZ-F1* and induces *E75A* and *E75B* (Figure 1), either of which is sufficient to repress *diap2* transcription (Figures 2B and 8). It is possible that *E75A* and *E75B* exert this repressive function in a redundant manner. This proposal is consistent with genetic studies of the *E75* locus that have revealed internal functional redundancy between *E75* isoforms (I. Johnson and D. Garza, personal communication; W. Segraves, personal communication). This functional redundancy also complicates the use of specific *E75* mutants as a means of determining their role in *diap2* transcriptional regulation. Efforts are currently underway to use *E75* null alleles to determine the phenotypes associated with a complete loss of all *E75* functions. In addition, it is unclear how E75A and E75B act as repressors. E75A could function like its mammalian homolog, Rev-Erbα, by recruiting a corepressor to inhibit target gene expression (Zamir et al., 1997). In contrast, the E75B isoform does not have a DNA-binding domain and, thus, must exert its effects through dimerization with another transcription factor, most likely a nuclear receptor. Ectopic expression studies have shown that E75B can dimerize with the DHR3 orphan nuclear receptor and thereby block the DHR3 transactivation function (White et al., 1997). DHR3, however, is not present in the salivary glands of late prepupae, and thus the partner for E75B function at this stage remains unknown (White et al., 1997). An intriguing possibility is that E75B may repress *diap2* by interacting with βFTZ-F1, switching this transcription factor from an activator to a repressor and thereby defining the duration of *diap2* expression.

Although *diap2* mutants have not yet been characterized, the timing of *diap2* expression immediately before *rpr* and *hid* induction in doomed salivary glands supports the model that *diap2* functions as an inhibitor of cell death in this tissue. This is consistent with the 2–3 hr delay in the first signs of salivary gland cell death relative to *rpr* and *hid* induction—a delay that is not seen in larval midguts where *diap1* and *diap2* are not expressed (Jiang et al., 1997; C. J., unpublished data). In addition, ectopic expression of *diap2* can effectively block *rpr* or *hid*-triggered apoptosis in adult eyes, supporting its role as a death inhibitor (Hay et al., 1995). We propose that *diap2* functions in the salivary glands as a checkpoint to ensure that these cells will only die in response to the strong apoptotic stimuli provided by both *rpr* and *hid* expression. It is interesting to note that IAPs are also induced in mammalian cells after treatment with tumor necrosis factor α (TNFα), suggesting that they may have a similar function in regulating apoptosis triggered by other death-causing agents (Wang et al., 1998).

rpr Transcription Is Induced Directly by the Ecdysone–Receptor Complex

Three lines of evidence indicate that *rpr* transcription is induced directly by the EcR/USP ecdysone receptor. First, *rpr* mRNA is first detected in staged salivary glands immediately after the prepupal pulse of ecdysone (Figure 1). Second, *rpr* transcription can be induced by ecdysone in the absence of protein synthesis, defining it as a primary response to the hormone (Figure 3). Third, 1.3 kb of the *rpr* promoter is sufficient for proper temporal regulation in vivo, and this region contains a single EcR/USP binding site that is essential for promoter activity in vivo (Figures 5 and 7). Taken together, these results provide strong evidence that the ecdysone receptor complex directly induces *rpr* transcription through at least one EcRE in the *rpr* promoter. This is consistent with genetic studies of the ecdysone receptor that have indicated that EcR and USP are required for salivary gland cell death during metamorphosis (Bender et al., 1997; Hall and Thummel, 1998).

Given that *rpr* expression is necessary and sufficient for programmed cell death (White et al., 1994, 1996), we propose that our studies provide a direct link between the steroid signal and a programmed cell death response. Although steroid hormones and thyroid hormone are known to be critical regulators of programmed cell death in invertebrates and vertebrates, little is known about the molecular mechanisms by which these signals are transduced into an apoptotic response. The steroid-triggered destruction of the larval salivary gland during *Drosophila* metamorphosis provides an ideal model system for defining these gene networks.

It should be noted, however, that there is as yet no definitive proof that *rpr* and *hid* are essential for salivary gland cell death. This would require the production of *Df(3L)H99* salivary gland clones—a method that is technically very difficult, if not impossible, in polytene larval tissues. We are currently attempting to develop new approaches for functional studies of early lethal mutations in the salivary glands. Nevertheless, the tight temporal and functional correlations shown in this paper

and our earlier publication (Jiang et al., 1997) strongly argue that *rpr* and *hid* play a central role in directing the destruction of the larval salivary glands during the onset of metamorphosis.

The Ecdysone-Induced BR-C and E74A Transcription Factors Regulate *rpr* and *hid* Transcription

Although *rpr* transcription is induced directly by the ecdysone–receptor complex, the levels of *rpr* mRNA are delayed and reduced in the absence of protein synthesis (Figure 3). Moreover, *hid* mRNA cannot be detected under these conditions. These observations indicate that ecdysone-induced transcription factors work together with the ecdysone–receptor complex to direct appropriate *rpr* and *hid* expression in doomed larval salivary glands. Our genetic studies have identified both the *BR-C* and *E74A* as essential components in this pathway (Figure 8).

The *BR-C* is required for both *rpr* and *hid* transcription (Figures 4A and 8) as well as salivary gland cell death (Restifo and White, 1992; Zhimulev et al., 1995). This is consistent with the essential role of the *BR-C* in regulating late ecdysone-inducible puffs in the larval salivary gland polytene chromosomes (Belyaeva et al., 1981). Furthermore, the *L71* late genes are not expressed in rbp^5 mutant salivary glands, and the Z1 isoform of the *BR-C*, which encodes the *rbp* function, has been shown to directly regulate *L71–6* transcription (Guay and Guild, 1991; Crossgrove et al., 1996).

Given the dramatic effect of the *BR-C* rbp^5 mutation on *rpr* expression, we attempted to determine whether the *BR-C* might directly regulate *rpr* transcription. We found that the 1.3 kb *rpr* promoter is not induced in rbp^5 mutant salivary glands, indicating that the *BR-C* exerts its regulatory effects through these *cis*-acting sequences (data not shown). Several strong Z1 binding sites were identified in the 1.3 kb *rpr* promoter fragment by DNase I footprinting. Mutating all of these sites, however, had no effect on the ability of the 1.3 kb fragment to direct *lacZ* induction in the salivary glands of late prepupae (data not shown). Based on this result, we conclude that the *BR-C* indirectly regulates *rpr* expression through one or more transcription factors that are dependent on the rbp^+ function (Figure 8). It will be interesting to determine if *hid* transcription is also indirectly regulated by the *BR-C* and whether any common features between the *rpr* and *hid* regulatory elements indicate what factor(s) might be mediating this control.

Although *E74A* mutants also display defects in salivary gland cell death, this phenotype is not as penetrant as the rbp^5 mutant phenotype. Only approximately 20% of the larval salivary glands in *E74A* mutant pupae persist until 24 hr after puparium formation. Consistent with this partially penetrant cell death defect, the levels of *hid* transcription are significantly reduced in *E74A* mutant salivary glands, while *rpr* transcription remains unaffected (Figure 4B). This observation implicates *E74A* as a critical inducer of *hid* transcription (Figure 8). It also provides further evidence that *rpr* and *hid* are regulated by different mechanisms, consistent with the distinct patterns of *rpr* and *hid* expression observed during embryogenesis (White et al., 1994; Grether et al., 1995) and the inhibition of HID activity, but not RPR or GRIM, by the RAS pathway (Bergmann et al., 1998a; Kurada and White, 1998). The distinct modes for regulating RPR, HID, and GRIM allow the organism to integrate a wide range of different apoptotic signals to precisely control the patterns of cell death during development.

Our *E74A* genetic studies also suggest that both RPR and HID must be expressed in the salivary glands to effectively trigger the death response. A similar mode of regulation has been demonstrated in the midline cells of the embryonic central nervous system, where both RPR and HID function together in a cooperative manner to direct cell death (Zhou et al., 1997). RPR and HID may exert slightly different functions in directing the destruction of the salivary glands, where the major function of RPR could be to antagonize the repressive effect of DIAP2 thereby increasing the sensitivity of the glands to the apoptotic effect of HID. A similar model has been proposed by Kurada and White (1998) to explain the synergistic activation of cell death by *rpr* and *hid* and their distinct modes of regulation.

Future Directions

Larval tissues are destroyed during *Drosophila* metamorphosis in a highly stage-specific manner. For example, the ecdysone pulse that triggers puparium formation also induces the coordinate induction of *rpr* and *hid* in the larval midguts, triggering the destruction of this tissue (Jiang et al., 1997). In contrast, the salivary glands survive this pulse of ecdysone and do not initiate the death program until after the next ecdysone pulse, approximately 10–12 hr after puparium formation. Interestingly, EcR/USP, BR-C, and E74A are all present in the salivary glands of newly formed prepupae, yet *rpr* and *hid* are not induced at this time (Figure 1). Clearly, there must be other critical regulators of *rpr* and *hid* transcription that determine the stage specificity of this response in the salivary glands. We are using two approaches to understand this level of control. First, we are studying the regulation of larval midgut cell death in an effort to compare and contrast this response with that seen in the salivary glands approximately 12 hr later. Second, we are searching for factors that are induced by ecdysone in a stage-specific manner in the salivary glands, in response to the prepupal pulse of ecdysone that triggers cell death. One of these factors is encoded by the *E93* early gene (Baehrecke and Thummel, 1995). *E93* is induced directly by ecdysone in late prepupal salivary glands but shows no response to the same signal several hours earlier, in newly formed prepupae. Both loss-of-function and gain-of-function genetic studies indicate that *E93* is an essential regulator of salivary gland cell death, defining this gene as a critical factor that contributes to the stage specificity of this response (C.-Y. Lee and E. H. Baehrecke, personal communication). An effort is also underway to identify other factors that function like *E93* to determine the appropriate timing of salivary gland cell death. Finally, the *rpr* and *hid* promoters provide an opportunity to work backward, in an effort to identify novel factors that contribute to the stage specificity of their regulation. By identifying the ecdysone-regulated factors that coordinate the death-specific expression of *rpr* and *hid*, we should be able

to establish a molecular basis for the steroid-triggered destruction of larval tissues during insect metamorphosis. These studies will provide insight into how hormonal signals are refined into distinct stage-specific biological responses during development as well as provide a framework for understanding how steroid hormones control programmed cell death in other higher organisms.

Experimental Procedures

Fly Stocks

FTZ-F1^{17} and the deficiency that removes the *FTZ-F1* locus, *Df(3L)Cat*^{DH104}, have been described by Broadus et al. (1999). These chromosomes were maintained over the balancer chromosome *TM6B Tb*. β*FTZ-F1* mutant prepupae were generated by crossing *FTZ-F1*^{17}*/TM6B* females with *Df(3L)Cat*^{DH104}*/TM6B* males and were identified by their *Tb*$^{+}$ phenotype. The *rbp*^{5} mutant chromosome (Belyaeva et al., 1980) was maintained over a *Binsn* balancer. Mutant males (*y rbp*^{5}/Y) were distinquished from their wild-type siblings (*y rbp*^{5}/+ or *Binsn*/Y) on the basis of their yellow mouth hooks at puparium formation. *E74*$^{P[neo]}$ and the deficiency that removes the *E74* locus, *Df(3L)st-81k19*, have been described by Fletcher et al. (1995). These chromosomes were maintained over the balancer chromosome *TM6B Hu e Tb ca*. *E74A* mutant prepupae and pupae were generated by crossing *E74*$^{P[neo]}$*TM6B* females with *Df(3L)st-81k19/TM6B* males and were identified by their *Tb*$^{+}$ phenotype.

Developmental Staging

Late third instar larvae were staged by growing them on food supplemented with 0.1% bromophenol blue, as described (Andres and Thummel, 1994). Blue gut mid–third instar larvae are referred as −18 hr relative to puparium formation, partially clear gut late third instar larvae are referred to as −8 hr, and clear gut late third instar larvae are referred to as −4 hr. Prepupae and pupae are staged in hours relative to puparium formation.

Northern Blot Hybridization

Total RNA isolated from dissected or cultured salivary glands was fractionated by formaldehyde gel electrophoresis and transferred to nylon membranes as described (Andres et al., 1993). The blot was sequentially hybridized with each radioactive probe and stripped for rehybridization. β*FTZ-F1*, *BR-C*, *E74A*, *E75A*, and *E75B* probes were prepared as described in Andres et al. (1993). *rpr*, *hid*, and *diap2* probes were prepared as described in Jiang et al. (1997).

In Vitro Culture of Larval Salivary Glands

Canton-S animals were synchronized at puparium formation and allowed to develop at 25°C for 10 hr. They were dissected in Schneider's *Drosophila* medium (GIBCO-BRL) and cultured in this medium containing either 5×10^{-6} M 20-hydroxyecdysone (Sigma) or both 5×10^{-6} M 20-hydroxyecdysone and 7.5×10^{-5} M cycloheximide (Sigma) for 0, 2, or 4 hr as described (Woodard et al., 1994). RNA was extracted from the cultured salivary glands and analyzed by Northern blot hybridization.

Deletion Analysis of the *rpr* Promoter

DNA fragments from the *rpr* promoter were derived from an 11 kb EcoRI genomic fragment in pNR11, described by Nordstrom et al. (1996). To facilitate fusion to the *lacZ* reporter gene, a KpnI site was introduced 125 bp downstream from the *rpr* transcription start site by site-directed mutagenesis. Four different restriction fragments were then excised from this plasmid and inserted into the pCasper-AUG-*lacZ* P element vector: a 9.6 kb EcoRI-KpnI fragment, a 6.2 kb BglII-KpnI fragment, a 4.0 kb NdeI-KpnI fragment, and a 1.3 kb EcoRV-KpnI fragment. These constructs carry different lengths of DNA upstream from the *rpr* transcription start site and 125 bp of the *rpr* 5′ untranslated region fused to the *lacZ* reporter gene. The four P element constructs were introduced into *y w*^{67} flies by P element–mediated germline transformation (Rubin and Spradling, 1982), and *lacZ* expression in the salivary glands of each transgenic line was determined by Northern blot hybridization.

DNA Binding Assays

EcR and USP proteins were synthesized in vitro using the TNT Coupled Reticulocyte Lysate System (Promega). Parallel reactions performed in the presence of [^{35}S]methionine demonstrated that EcR and USP are synthesized with similar efficiency. DNA binding assays were carried out in a total volume of 20 μl containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 200 mM BSA, 5% (v/v) glycerol, 1 μg poly(dI-dC) (Sigma), ~0.5 ng ^{32}P-labeled probe, and 1 μl of in vitro translated EcR and/or USP protein. After 20 min at 25°C, assay mixtures were loaded onto 6% nondenaturing polyacrylamide gels and run for 75 min at 200 V in 0.5× TBE. After electrophoresis, the gels were dried for autoradiography.

The following double-stranded oligonucleotides were used as ^{32}P-labeled probes or as unlabeled oligonucleotides for DNA binding competition: *hsp27* EcRE, TTATTGGACAAGTGCATTGAACCCTTG TCTCTCC/GGAGAGACAAGGGTTCAATGCACTTGTCCAAT; *rpr* EcRE, GAACAAAGTGAACGAACTCGAAA/TTTCGAGTTCGTTCACTTTGTT; mutant *rpr* EcRE, GACACCAGAACAAGGCCCACGAACTCGAAAA/TTT TCGAGTTCGTCACCTTTGTTCTGGTGT.

The oligonucleotides were labeled with [α-^{32}P]dATP or [α-^{32}P]dCTP by filling in the ends using the exo(−) Klenow fragment of DNA polymerase (Stratagene).

Mutagenesis of the *rpr* EcRE

The EcR/USP binding site in the 1.3 kb *rpr* promoter fragment was mutated by PCR-mediated mutagenesis. The wild-type 317 bp HincII-KpnI fragment was used as a template for a series of sequencial PCR reactions that introduced four point mutations into the EcR/USP binding site. A pair of oligonucleotides were used for this mutagenesis: 5′-GACACCAGAACAAGGCCCACGAACTCGAAA-3′ and 5′-TTTTCGAGTTCGTGGGCCTTGTTCTGGTGT-3′. The sequence of the mutant 317 bp fragment was confirmed by fluorescent-tagged DNA sequencing (ABI). The mutant 317 bp fragment was then used to replace the wild-type fragment in the 1.3 kb *rpr* promoter region. This mutant promoter fragment was then inserted into pCasper-AUG-lacZ as described above, and germline transformants were generated.

Ectopic Expression of *E75A* and *E75B*

The P[*hs-E75A*] construct was generated by inserted the *E75A* coding region into the BamHI site of the pABAL P element vector (Zeng et al., 1993). The P[*hs-E75B*] construct was generated by inserting the *E75B* coding region between the EcoRI and BamHI sites of pABAL. These constructs were introduced into *w*^{1118} flies by P element–mediated germline transformation (Rubin and Spradling, 1982).

To ectopically express *E75A* and *E75B*, control *w*^{1118} animals, P[*hs-E75A*] and P[*hs-E75B*] transformants were synchronized at puparium formation and allowed to develop at 25°C for 7.5 hr. They were then incubated in a 37°C water bath for 30 min and allowed to recover at 25°C for 2 hr, after which their salivary glands were dissected. For the non-heat-treated controls, animals were staged at puparium formation and allowed to develop at 25°C for 10 hr before salivary glands were dissected. RNA was then extracted from the salivary glands and analyzed by Northern blot hybridization.

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References

Abrams, J.M. (1999). An emerging blueprint for apoptosis in *Drosophila*. Trends Cell Biol. *9*, 435–440.

Adelmant, G., Begue, A., Stehelin, D., and Laudet, V. (1996). A functional Rev-erbα responsive element located in the human Rev-erbα promoter mediates a repressing activity. Proc. Natl. Acad. Sci. USA *93*, 3553–3558.

Andres, A.J., and Thummel, C.S. (1994). Methods for quantitative analysis of transcription in larvae and prepupae. In *Drosophila melanogaster*: Practical Uses in Cell and Molecular Biology, L.S.B. Goldstein and E.A. Fyrberg, ed. (New York: Academic Press), pp. 565–573.

Andres, A.J., Fletcher, J.C., Karim, F.D., and Thummel, C.S. (1993). Molecular analysis of the initiation of insect metamorphosis: a comparative study of *Drosophila* ecdysteroid-regulated transcription. Dev. Biol. *160*, 388–404.

Ashburner, M. (1974). Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. II. The effects of inhibitors of protein synthesis. Dev. Biol. *39*, 141–157.

Baehrecke, E.H., and Thummel, C.S. (1995). The *Drosophila E93* gene from the 93F early puff displays stage- and tissue-specific regulation by 20-hydroxyecdysone. Dev. Biol. *171*, 85–97.

Belyaeva, E.S., Aizenzon, M.G., Semeshin, V.F., Kiss, I.I., Koezka, K., Baritcheva, E.M., Gorelova, T.D., and Zhimulev, I.F. (1980). Cytogenetic analysis of the 2B3–4 to 2B11 region of the X-chromosome of *Drosophila melanogaster*. I. Cytology of the region and mutant complementation groups. Chromosoma *81*, 281–306.

Belyaeva, E.S., Vlassova, I.E., Biyasheva, Z.M., Kakpakov, V.T., Richards, G., and Zhimulev, I.F. (1981). Cytogenetic analysis of the 2B3–4–2B11 region of the X chromosome of *Drosophila melanogaster*. II. Changes in 20-OH ecdysone puffing caused by genetic defects of puff 2B5. Chromosoma *84*, 207–219.

Belyaeva, E.S., Aizenzon, M.G., Kiss, I., Gorelova, T.D., Pak, S., Umbetova, G.H., Kramers, P.G.N., and Zhimulev, I.F. (1982). New mutants. Dros. Info. Serv. *58*, 184–190.

Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B., and Hogness, D.S. (1997). Drosophila ecdysone receptor mutations reveal functional differences among receptor isoforms. Cell *91*, 777–788.

Bergmann, A., Agapite, J., McCall, K., and Steller, H. (1998a). The *Drosophila* gene *hid* is a direct molecular target of Ras-dependent survival signaling. Cell *95*, 331–341.

Bergmann, A., Agapite, J., and Steller, H. (1998b). Mechanisms and control of programmed cell death in invertebrates. Oncogene *17*, 3215–3223.

Birnbaum, M.J., Clem, R.J., and Miller, L.K. (1994). An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. J. Virol. *68*, 2521–2528.

Broadus, J., McCabe, J.R., Endrizzi, B., Thummel, C.S., and Woodard, C.T. (1999). The *Drosophila* βFTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. Mol. Cell *3*, 143–149.

Burtis, K.C., Thummel, C.S., Jones, C.W., Karim, F.D., and Hogness, D.S. (1990). The *Drosophila* 74EF early puff contains *E74*, a complex ecdysone-inducible gene that encodes two *ets*-related proteins. Cell *61*, 85–99.

Chen, P., Nordstrom, W., Gish, B., and Abrams, J.M. (1996). *grim*, a novel cell death gene in *Drosophila*. Genes Dev. *10*, 1773–1782.

Clem, R.J., and Miller, L.K. (1994). Control of programmed cell death by the baculovirus genes p35 and iap. Mol. Cell. Biol. *14*, 5212–5222.

Crook, N.E., Clem, R.J., and Miller, L.K. (1993). An apoptosis-inhibiting baculovirus gene with a zinc finger–like motif. J. Virol. *67*, 2168–2174.

Crossgrove, K., Bayer, C.A., Fristrom, J.W., and Guild, G.M. (1996). The *Drosophila Broad-Complex* early gene directly regulates late gene transcription during the ecdysone-induced puffing cascade. Dev. Biol. *180*, 745–758.

DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W., and Guild, G.M. (1991). The *Drosophila Broad-Complex* encodes a family of related proteins containing zinc fingers. Genetics *129*, 385–397.

Dorstyn, L., Colussi, P.A., Quinn, L.M., Richardson, H., and Kumar, S. (1999). DRONC, an ecdysone-inducible *Drosophila* caspase. Proc. Natl. Acad. Sci. USA *96*, 4307–4312.

Draizen, T.A., Ewer, J., and Robinow, S. (1999). Genetic and hormonal regulation of the death of peptidergic neurons in the *Drosophila* central nervous system. J. Neurobiol. *38*, 455–465.

Duckett, C.S., Nava, V.E., Gedrich, R.W., Clem, R.J., Van Dongen, J.L., Gilfillan, M.C., Shiels, H., Hardwick, J.M., and Thompson, C.B. (1996). A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. EMBO J. *15*, 2685–2694.

Emery, I.F., Bedian, V., and Guild, G.M. (1994). Differential expression of Broad-Complex transcription factors may forecast tissue-specific developmental fates during *Drosophila* metamorphosis. Development *120*, 3275–3287.

Evans-Storms, R.B., and Cidlowski, J.A. (1995). Regulation of apoptosis by steroid hormones. J. Steroid Biochem. Mol. Biol. *53*, 1–6.

Fletcher, J.C., and Thummel, C.S. (1995). The ecdysone-inducible *Broad-Complex* and *E74* early genes interact to regulate target gene transcription and *Drosophila* metamorphosis. Genetics *141*, 1025–1035.

Fletcher, J.C., Burtis, K.C., Hogness, D.S., and Thummel, C.S. (1995). The *Drosophila E74* gene is required for metamorphosis and plays a role in the polytene chromosome puffing response to ecdysone. Development *121*, 1455–1465.

Goyal, L., McCall, K., Agapite, J., Hartwieg, E., and Steller, H. (2000). Induction of apoptosis by *Drosophila reaper*, *hid*, and *grim* through inhibition of IAP function. EMBO J. *19*, in press.

Grether, M.E., Abrams, J.M., Agapite, J., White, K., and Steller, H. (1995). The *head involution defective* gene of *Drosophila melanogaster* functions in programmed cell death. Genes Dev. *9*, 1694–1708.

Guay, P.S., and Guild, G.M. (1991). The ecdysone-induced puffing cascade in *Drosophila* salivary glands: a *Broad-Complex* early gene regulates intermolt and late gene transcription. Genetics *129*, 169–175.

Hall, B.L., and Thummel, C.S. (1998). The RXR homolog Ultraspiracle is an essential component of the *Drosophila* ecdysone receptor. Development *125*, 4709–4717.

Harding, H.P., and Lazar, M.A. (1995). The monomer-binding orphan receptor Rev-Erb represses transcription as a dimer on a novel direct repeat. Mol. Cell. Biol. *15*, 4791–4802.

Hawkins, C.J., Wang, S.L., and Hay, B.A. (1999). A cloning method to identify caspases and their regulators in yeast: identification of *Drosophila* IAP1 as an inhibitor of the *Drosophila* caspase DCP-1. Proc. Natl. Acad. Sci. USA *96*, 2885–2890.

Hay, B.A., Wassarman, D.A., and Rubin, G.M. (1995). *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell *83*, 1253–1262.

Henrich, V.C., Szekely, A.A., Kim, S.J., Brown, N.E., Antoniewski, C., Hayden, M.A., Lepesant, J.-A., and Gilbert, L.I. (1994). Expression and function of the *ultraspiracle* (*usp*) gene during development of *Drosophila melanogaster*. Dev. Biol. *165*, 38–52.

Jacobson, M.D., Weil, M., and Raff, M.C. (1997). Programmed cell death in animal development. Cell *88*, 347–354.

Jiang, C., Baehrecke, E.H., and Thummel, C.S. (1997). Steroid regulated programmed cell death during *Drosophila* metamorphosis. Development *124*, 4673–4683.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Brit. J. Cancer *26*, 239–257.

Kiess, W., and Gallaher, B. (1998). Hormonal control of programmed cell death/apoptosis. Eur. J. Endocrinol. *138*, 482–491.

Kiss, I., Beaton, A.H., Tardiff, J., Fristrom, D., and Fristrom, J.W. (1988). Interactions and developmental effects of mutations in the *Broad-Complex* of *Drosophila melanogaster*. Genetics *118*, 247–259.

Koelle, M.R. (1992). Molecular analysis of the *Drosophila* ecdysone receptor complex. PhD thesis, Stanford University, Stanford, CA.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., and Hogness, D.S. (1991). The Drosophila EcR gene encodes an

ecdysone receptor, a new member of the steroid receptor superfamily. Cell *67*, 59–77.

Kurada, P., and White, K. (1998). Ras promotes cell survival in *Drosophila* by downregulating hid expression. Cell *95*, 319–329.

Lehmann, M., and Korge, G. (1995). Ecdysone regulation of the *Drosophila Sgs-4* gene is mediated by the synergistic action of ecdysone receptor and SEBP 3. EMBO J. *14*, 716–726.

Lockshin, R.A., and Williams, C.M. (1965). Programmed cell death. II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkworms. J. Insect Physiol. *11*, 803–809.

Nordstrom, W., Chen, P., Steller, H., and Abrams, J.M. (1996). Activation of the reaper gene during ectopic cell killing in *Drosophila*. Dev. Biol. *180*, 213–226.

Restifo, L.L., and White, K. (1992). Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of internal tissues in *Drosophila*: salivary glands, muscle, and gut. Roux's Arch. Dev. Biol. *201*, 221–234.

Riddiford, L.M. (1993). Hormones and *Drosophila* development. In The Development of *Drosophila melanogaster*, Volume II, M. Bate and A. Martinez Arias, ed. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press), pp. 899–940.

Riddihough, G., and Pelham, H.R.R. (1987). An ecdysone response element in the *Drosophila hsp27* promoter. EMBO J. *6*, 3729–3734.

Robertson, C.W. (1936). The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the principal morphological changes. J. Morphol. *59*, 351–399.

Robinow, S., Draizen, T.A., and Truman, J.W. (1997). Genes that induce apoptosis: transcriptional regulation in identified, doomed neurons of the *Drosophila* CNS. Dev. Biol. *190*, 206–213.

Rubin, G.M., and Spradling, A.C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. Science *218*, 348–353.

Rudin, C.M., and Thompson, C.B. (1997). Apoptosis and disease: regulation and clinical relevance of programmed cell death. Annu. Rev. Med. *48*, 267–281.

Russell, S., and Ashburner, M. (1996). Ecdysone-regulated chromosome puffing in *Drosophila melanogaster*. In Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells, L.I. Gilbert, J.R. Tata, and B.G. Atkinson, ed. (New York: Academic Press), pp. 109–144.

Segraves, W.A., and Hogness, D.S. (1990). The *E75* ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. Genes Dev. *4*, 204–219.

Tata, J.R. (1966). Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. Dev. Biol. *13*, 77–94.

Thomas, H.E., Stunnenberg, H.G., and Stewart, A.F. (1993). Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. Nature *362*, 471–475.

Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science *267*, 1456–1462.

Thummel, C.S. (1996). Flies on steroids—*Drosophila* metamorphosis and the mechanisms of steroid hormone action. Trends Genet. *12*, 306–310.

Urness, L.D., and Thummel, C.S. (1995). Molecular analysis of a steroid-induced regulatory hierarchy: The *Drosophila* E74A protein directly regulates *L71–6* transcription. EMBO J. *14*, 6239–6246.

Vaux, D.L., and Korsmeyer, S.J. (1999). Cell death in development. Cell *96*, 245–254.

Vucic, D., Kaiser, W.J., Harvey, A.J., and Miller, L.K. (1997). Inhibition of reaper-induced apoptosis by interaction with inhibitor of apoptosis proteins (IAPs). Proc. Natl. Acad. Sci. USA *94*, 10183–10188.

Vucic, D., Kaiser, W.J., and Miller, L.K. (1998). Inhibitor of apoptosis proteins physically interact with and block apoptosis induced by *Drosophila* proteins HID and GRIM. Mol. Cell. Biol. *18*, 3300–3309.

Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V., and Baldwin, A.S., Jr. (1998). NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science *281*, 1680–1683.

Wang, S.L., Hawkins, C.J., Yoo, S.J., Müller, H.-A., and Hay, B.A. (1999). The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. Cell *98*, 453–463.

White, K., Grether, M.E., Abrams, J.M., Young, L., Farrell, K., and Steller, H. (1994). Genetic control of programmed cell death in *Drosophila*. Science *264*, 677–683.

White, K., Tahaoglu, E., and Steller, H. (1996). Cell killing by the *Drosophila* gene *reaper*. Science *271*, 805–807.

White, K.P., Hurban, P., Watanabe, T., and Hogness, D.S. (1997). Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. Science *276*, 114–117.

Woodard, C.T., Baehrecke, E.H., and Thummel, C.S. (1994). A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone. Cell *79*, 607–615.

Yao, T.P., Segraves, W.A., Oro, A.E., McKeown, M., and Evans, R.M. (1992). *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell *71*, 63–72.

Zamir, I., Dawson, J., Lavinsky, R.M., Glass, C.K., Rosenfeld, M.G., and Lazar, M.A. (1997). Cloning and characterization of a corepressor and potential component of the nuclear hormone receptor repression complex. Proc. Natl. Acad. Sci. USA *94*, 14400–14405.

Zeng, W., Andrew, D.J., Mathies, L.D., Horner, M.A., and Scott, M.P. (1993). Ectopic expression and function of the *Antp* and *Scr* homeotic genes: the N terminus of the homeodomain is critical to functional specificity. Development *118*, 339–352.

Zhimulev, I.F., Belyaeva, E.S., Mazina, O.M., and Balasov, M.L. (1995). Structure and expression of the *BR-C* locus in *Drosophila melanogaster* (Diptera: Drosophilidae). Eur. J. Entomol. *92*, 263–270.

Zhou, L., Schnitzler, A., Agapite, J., Schwartz, L.M., Steller, H., and Nambu, J.R. (1997). Cooperative functions of the *reaper* and *head involution defective* genes in the programmed cell death of *Drosophila* central nervous system midline cells. Proc. Natl. Acad. Sci. USA *94*, 5131–5136.