

Ecdysone-induced expression of the caspase DRONC during hormone-dependent programmed cell death in *Drosophila* is regulated by Broad-Complex

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The steroid hormone ecdysone regulates both cell differentiation and cell death during insect metamorphosis, by hierarchical transcriptional regulation of a number of genes, including the *Broad-Complex* (*BR-C*), the zinc finger family of transcription factors. These genes in turn regulate the transcription of a number of downstream genes. DRONC, a key apical caspase in *Drosophila*, is the only known caspase that is transcriptionally regulated by ecdysone during development. We demonstrate that *dronc*

gene expression is ablated or reduced in *BR-C* mutant flies. Using RNA interference in an ecdysone-responsive *Drosophila* cell line, we show that DRONC is essential for ecdysone-mediated cell death, and that *dronc* upregulation in these cells is controlled by BR-C. Finally, we show that the *dronc* promoter has BR-C interaction sites, and that it can be transactivated by a specific isoform of BR-C. These results indicate that BR-C plays a key role in ecdysone-mediated caspase regulation.

Introduction

Programmed cell death is essential to delete unwanted or superfluous cells in metazoans. Recent studies suggest that the core cell death machinery is highly conserved and is present in all metazoan cells (for review see Vaux and Korsmeyer, 1999). Although both pro-survival and pro-apoptotic components of the cell death machinery are present constitutively within a cell, a balance between pro- and anti-death factors appears to regulate the apoptotic process. Various signals such as cytotoxic insults, hormones, and growth factors regulate the activation of the death program by controlling the balance between pro- and anti-death factors (Strasser et al., 2000). Although the composition of the cell death effector machinery is now largely understood, how the upstream signals communicate with the core components of the machinery remains poorly defined.

Many hormones are known to regulate cell survival and cell death both in embryonic and adult tissues. One of the important models of hormone-regulated cell death is *Drosophila melanogaster*, in which a single steroid hormone, 20-hydroxy ecdysone, regulates programmed cell death to remove obso-

lete larval tissues (for review see Thummel, 1996; Baehrecke, 2000). Pulses of ecdysone are produced at various times during fly development and regulate cell proliferation, differentiation, and death in a temporally and spatially controlled manner (Riddiford, 1993). An ecdysone pulse toward the end of the larval stage signals puparium formation. A second pulse ~12 h later initiates head eversion. During the course of metamorphosis, obsolete larval tissues such as salivary glands and midgut are deleted, and progenitor cells give rise to adult tissues (Russell and Ashburner, 1996; Thummel, 1996; Baehrecke, 2000). Cell death in larval midgut begins at puparium formation in response to the late larval pulse of ecdysone, whereas the salivary glands undergo removal ~15 h later in response to the second pulse of ecdysone (Jiang et al., 1997).

Ecdysone binds to its heterodimeric EcR/Usp receptor and transcriptionally regulates a number of primary response genes; these include the *Broad-Complex* (*BR-C*),* *E74*, and *E75* (Russell and Ashburner, 1996; Thummel, 1996; Baehrecke, 2000). All of these genes encode transcription regulators. For example, *BR-C* encodes a family of zinc finger transcription factors (DiBello et al., 1991), *E74* encodes

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*Abbreviations used in this paper: BR-C, Broad-Complex; dsRNA, double-stranded RNA; EMSA, electrophoretic mobility shift analysis; FKH, Fork Head; RNAi, RNA interference.

ETS-like transcription factors (Burtis et al., 1990), and *E75* encodes orphan members of the nuclear receptor family (Seagraves and Hogness, 1990). In turn, these transcription factors regulate several secondary response genes. Ecdysone- and ecdysone-induced genes, including *βFTZ-F1*, *BR-C*, *E74*, *E75*, and *E93*, have been shown to play a role in ecdysone-mediated cell death in salivary gland and midgut (Thummel, 1996; Baehrecke, 2000). For example, *rpr* transcription in salivary glands is directly regulated by the EcR/Usp complex (Jiang et al., 2000). *BR-C* function also appears to be required for maximal *rpr* expression (Jiang et al., 2000). *BR-C* and *E74A* are required for the optimal induction of *hid* in salivary glands, whereas *βFTZ-F1* is required for the induction of *diap2* in late prepupal salivary glands, and *E75* represses this death inhibitor that precedes *rpr* and *hid* induction (Jiang et al., 2000). In salivary glands of the *E93* mutants *rpr*, *hid*, *dark*, and *dronc*, mRNA levels are severely reduced (Lee et al., 2000). These recent results suggest that ecdysone-mediated upregulation of death initiators such as *rpr*, *hid*, *dark*, and *dronc*, and downregulation of death inhibitors such as *diap1* and *diap2*, may be crucial to the synchronous removal of large number of cells in tissues such as salivary glands and midgut during *Drosophila* metamorphosis.

Cysteine proteases of the caspase family are key effectors of programmed cell death (for review see Kumar, 1999; Strasser et al., 2000). There are seven caspases in *Drosophila*, including DRONC, DRICE, DCP-1, DREDD, DECA, DAMM, and STRICA (Fraser and Evan, 1997; Song et al., 1997; Chen et al., 1998; Dorstyn et al., 1999a, 1999b; Doumanis et al., 2001; Harvey et al., 2001). Of these caspases, DRONC, DREDD, DCP-1, and DRICE have been shown to play a role in the execution of cell death (Fraser and Evan, 1997; Fraser et al., 1997; Song et al., 1997; Chen et al., 1998; McCall and Stellar, 1998; Dorstyn et al., 1999a, 1999b; Hawkins et al., 2000; for review see Kumar and Doumanis, 2000; Meier et al., 2000; Quinn et al., 2000). DRONC is a CED3/caspase-9-like, apical caspase containing a caspase recruitment domain that is believed to interact with the adaptor DARK (Rodriguez et al., 1999; Quinn et al., 2000). DRONC function is essential for developmental cell death in embryos, and it has been shown to be required for *rpr*-, *hid*-, and *grim*-mediated apoptosis in the fly eye (Hawkins et al., 2000; Meier et al., 2000; Quinn et al., 2000). DRONC interacts with and processes the effector caspase DRICE, suggesting that DRONC is an initiator caspase (Hawkins et al., 2000; Kumar and Doumanis, 2000; Meier et al., 2000). Although the biochemical mechanism of DRONC activation is not fully understood, recent studies demonstrate that DARK is necessary for the initial activation of DRONC (Dorstyn et al., 2002).

Our previous work has shown that *dronc* is transcriptionally regulated by ecdysone in salivary glands and midgut during larval-pupal metamorphosis (Dorstyn et al., 1999a, 1999b). A massive upregulation of *dronc* transcript occurring in late third instar larvae may facilitate its proximity induced activation (Dorstyn et al., 1999a, 1999b). One of the known factors regulating *dronc* transcription in salivary glands is *E93* (Lee et al., 2000); however, ecdysone-induced *dronc* upregulation may also be mediated by other factors. In the present paper, we demonstrate a key role for *BR-C* in *dronc* gene regulation.

Results

Ecdysone induces caspase activation and cell death in *l(2)mbn* cells

Previous studies have shown that ectopic expression of DRONC precursor causes cell death both in transgenic flies and in *Drosophila* cells lines, presumably by autoactivation of proDRONC (Dorstyn et al., 1999a; Hawkins et al., 2000; Meier et al., 2000; Quinn et al., 2000). In order to establish the importance of DRONC in ecdysone-mediated cell death, and to elucidate the mechanisms governing transcriptional upregulation of *dronc* in response to ecdysone, we used a hemocyte-derived *Drosophila* cell line *l(2)mbn*, which undergoes apoptosis in response to ecdysone treatment (Ress et al., 2000). As shown in Fig. 1 A, within 6 h of treatment of *l(2)mbn* cells with 10 μM ecdysone, >50% of the cells had undergone apoptosis, and by 48 h, almost all treated cells were lost. An analysis of extracts from *l(2)mbn* cells treated with ecdysone showed an increase in caspase activity. Interestingly, the activity on DRONC substrate, VDVAD-amc, became apparent within 6 h and did not increase thereafter. On the other hand, DEVD-amc cleavage activity, most of which is likely to be attributable to downstream caspases DRICE and DCP-1, was evident at 12 h and steadily increased thereafter (Fig. 1 A). This pattern of caspase activation is consistent with DRONC being an apical caspase responsible for the activation of downstream caspases.

Ecdysone induces *dronc* expression

Northern analysis revealed very low levels of *dronc* transcript in untreated *l(2)mbn* cells. Ecdysone treatment induced the expression of *dronc* within 2 h, reaching maximal levels at 12 h, and remained stable up to 48 h (Fig. 1 B). Immunoblot analysis using a DRONC antibody revealed that ecdysone treatment resulted in an increase in the levels of DRONC protein. Although the amount of DRONC precursor remained largely unchanged, a processed form of DRONC, representing active DRONC caspase, first became evident at 2 h and increased with time thereafter (Fig. 1 C; unpublished data). This increase in DRONC correlated with the enhanced caspase activity and ecdysone-mediated apoptosis observed in *l(2)mbn* cells. The results suggest that ecdysone-induced upregulation of *dronc* transcript results in increased intracellular DRONC protein; however, as DRONC precursor begins to accumulate, it is rapidly processed to generate active DRONC. A lag in the accumulation of DRONC protein, as compared to the *dronc* transcript, may reflect some translational regulation.

Because upregulation of DRONC and the accumulation of processed DRONC correlates with ecdysone-induced apoptosis, we wished to examine whether upregulation of DRONC alone can cause cell death. Therefore, we used a copper-inducible *dronc* expression construct, which was transfected into cells together with a *LacZ* reporter. Copper sulfate-mediated induction of *dronc* expression in cells transfected with increasing amounts of *dronc* expression construct showed a dose-dependent increase in dying cells (Fig. 1 D).

Our data demonstrate that *dronc* transcript accumulation is rapidly followed by accumulation and processing of DRONC protein, and that DRONC accumulation by over-

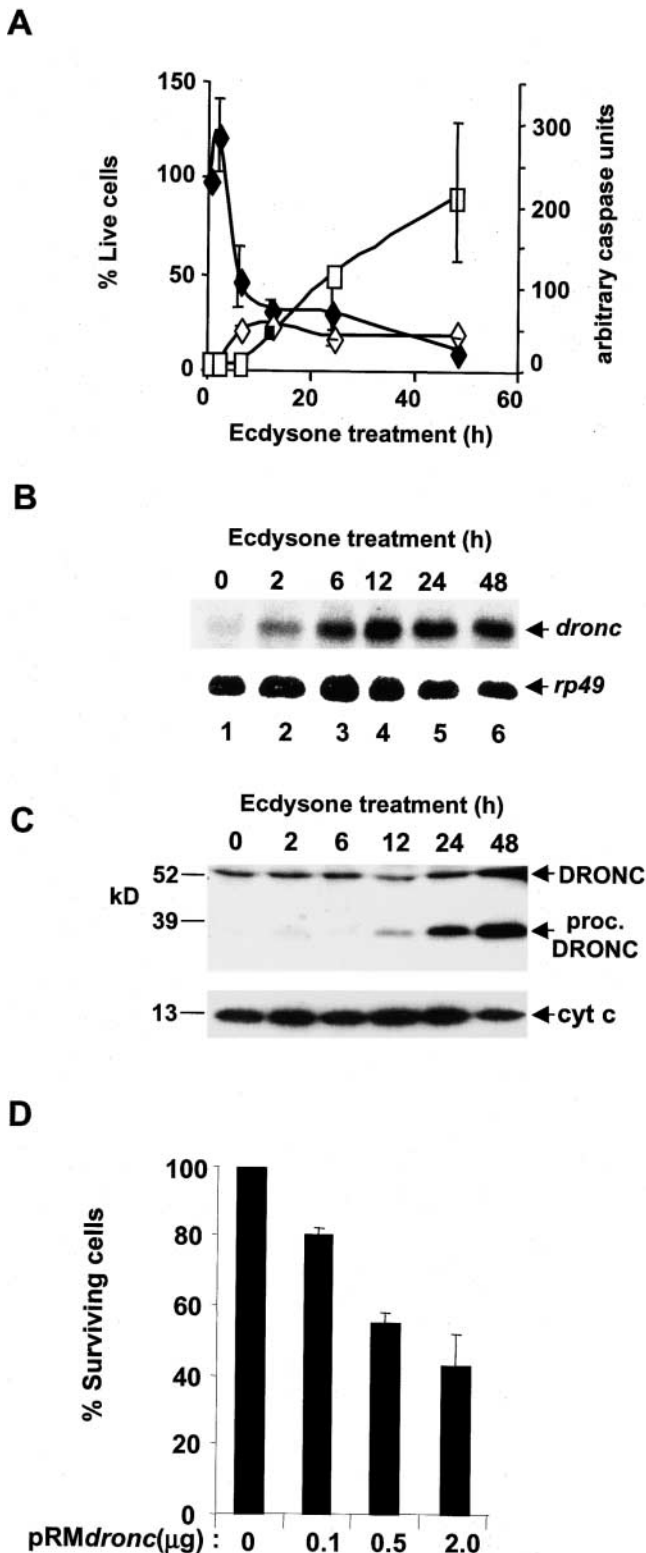


Figure 1. Ecdysone induces caspase-dependent cell death in *l(2)mbn* cells. (A) 1.5×10^6 cells were seeded in duplicate and treated with $10 \mu\text{M}$ ecdysone for indicated time. Cell viability (◆) was determined by Trypan blue exclusion. 50 μg of lysate was assayed for caspase activity using VDVAD-amc (◇) or DEVD-amc (□). Error bars represent SD. (B) Northern blot analysis was performed using total RNA derived from *l(2)mbn* cells treated with ecdysone for indicated time. *rp49* was used as a loading control. (C) Immunoblot analysis of DRONC protein. 50 μg of protein was electrophoresed, blotted onto PVDF membrane, and probed with an

expression can cause significant levels of apoptosis (Fig. 1). Thus, the simplest conclusion is that transcriptional regulation is a critical step in caspase activation, as full-length and processed DRONC accumulate relatively fast. However, the delay in accumulation of high levels of processed DRONC, and relatively slow and inefficient apoptosis induced by the overexpression of DRONC, indicate that a secondary regulatory step is likely involved. Indeed, ecdysone-mediated induction of the death activators *rpr*, *hid*, and *dark*, and down-regulation of death inhibitors *diap1* and *diap2* in salivary glands, is consistent with this model, as caspase regulation is known to be impacted by these molecules (Jiang et al., 2000; Lee et al., 2000).

Ecdysone-induced *dronc* expression is essential for cell death

The transcriptional upregulation and processing of DRONC in response to ecdysone clearly correlates with the ecdysone-mediated apoptosis of *l(2)mbn* cells. To examine whether DRONC expression and processing is necessary for apoptosis, we used RNA interference (RNAi) to ablate gene function (Hunter, 1999). Double-stranded RNA (dsRNA) corresponding to a 265-bp coding region of *dronc* was used alongside a negative control dsRNA designed for a 190-bp region of the murine Nedd4. Immunoblot analysis showed that *dronc* RNAi resulted in an ablation of DRONC protein expression, and thus, generation of active processed DRONC. On the other hand, the control RNAi had no significant effect on the levels of DRONC protein or DRONC processing, illustrating the specificity of *dronc* RNAi (Fig. 2 A). An examination of cells from the same experiment showed that *dronc* RNAi strongly suppressed ecdysone-mediated apoptosis of *l(2)mbn* cells, whereas the negative control, Nedd4 RNAi, had no effect on apoptosis (Fig. 2 B). Therefore, specific *dronc* ablation by RNAi effectively abrogates ecdysone-mediated cell death, indicating that DRONC is an essential mediator of ecdysone-induced apoptosis.

Ecdysone-inducible complexes are recruited to the *dronc* promoter and bind to BR-C consensus sites

Ecdysone-induced *dronc* transcription prompted the analysis of *dronc* regulation by the ecdysone receptor itself or ecdysone-induced transcription factors. Analysis of the proximal promoter revealed a cluster of potential BR-C binding sites from the region -7 to -176 bp from transcription start site (Fig. 3 A). It should be noted that previous studies have

anti-DRONC antibody. Full-length and processed (proc.) DRONC species are indicated. In long exposures of the blot, small amount of processed DRONC was also visible at 2 and 6 h (unpublished data). In the lower panel cytochrome c (cyt c) blot is shown as a loading control. (D) Overexpression of *dronc* induces cell death. 1.5×10^6 *l(2)mbn* cells were transfected with pRM*dronc* together with 500 ng of a *LacZ* expression construct (pIE *LacZ*). Total DNA was made up to 2.5 μg with empty vector. Expression was induced 24 h post-transfection by the addition of CuSO_4 to a final concentration of 0.7 mM. Loss of cells was determined 48 h postinduction by the ratio of β -galactosidase positive blue cells in copper-induced transfectants to the number of blue cells in uninduced cells, as previously described (Doumanis et al., 2001). Data (\pm SEM) are derived from two separate experiments performed in duplicate.

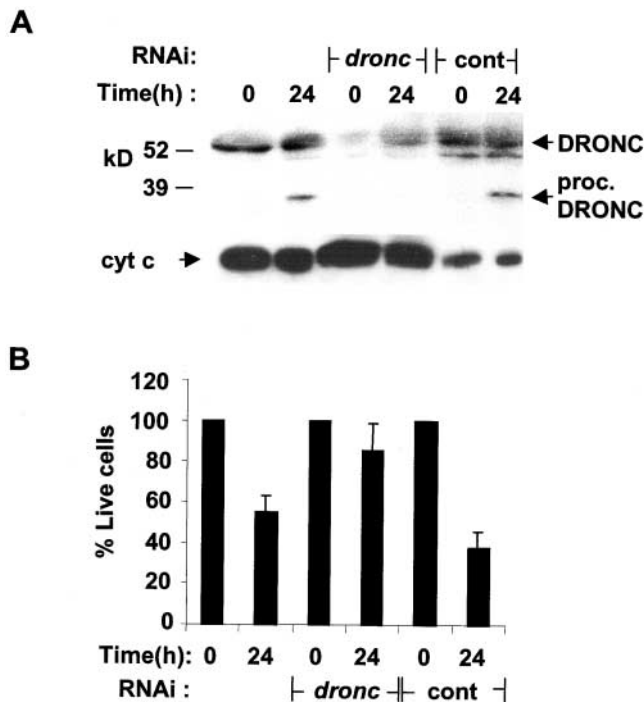


Figure 2. DRONC is essential for ecdysone-mediated cell death. (A) 2×10^6 *l(2)mbn* cells in Schneider media were exposed to 37 nM DRONC dsRNA or a negative control (cont) overnight. Cells were then treated with 10 μ M ecdysone for 24 h or left untreated (0). Cells lysates were immunoblotted with DRONC antibody or cytochrome c (cyt c) antibody as a loading control. Full length and processed (proc.) DRONC species are indicated. (B) Cells were treated with dsRNA and ecdysone in duplicate as in (A) and then counted using Trypan blue. Cell viability was determined as in Fig. 1. Error bars represent SD.

shown that BR-C sites are also binding sites for the transcription factor, Fork Head (FKH) (Renault et al., 2001). BR-C has four isoforms generated from one gene by alternatively spliced transcripts (DiBello et al., 1991). We analyzed the expression of BR-C proteins by immunoblotting of *l(2)mbn* cells treated with 10 μ M ecdysone for various lengths of time. For immunoblotting, a BR-C antibody which recognizes the NH₂-terminal region common to all four BR-C isoforms was used. Untreated *l(2)mbn* cells only contained one of the two, p64 and p57, BR-C Z2 species (Fig. 3 B). However, ecdysone treatment resulted in the appearance of the Z1/Z3 isoforms (both \sim 91 kD) within 2 h, followed by the Z4 isoform (118 kD). All isoforms showed a steady increase in their levels up to 24 h of ecdysone treatment, followed by a decline at 48 h (Fig. 3 B).

Electrophoretic mobility shift analysis (EMSA) using *l(2)mbn* cell nuclear extracts showed two distinct complexes binding to a 60-bp probe D4 which spans from -7 bp to -66 bp of the *dronc* promoter (Fig. 3 C). Treatment with ecdysone for 2 h showed no difference; however, at 6 h there was an increase in binding of one of the preexisting complexes (complex I). Also, *l(2)mbn* extracts prepared from cells treated with ecdysone for 6 h showed the appearance of slower and faster migrating complexes (complexes a and b, respectively), with the faster migrating complex being the most predominant. The same complexes were also seen for

probe D5 spanning from -57 to -116 and for D6 spanning from -117 to -176 . However, these probes displayed a lower affinity for the ecdysone-induced complexes (Fig. 3 C).

In order to gain further insight into the identity of the ecdysone-induced complexes, competition experiments were performed using cold oligonucleotides containing individual consensus sequences for all four BR-C isoforms. Consensus binding sites have been previously characterized by footprint analyses of recombinant BR-C isoforms binding to the *sgs-4* promoter (von Kalm et al., 1994). Nuclear extracts from cells treated with ecdysone for 6 h were used. Binding to the D4 probe resulted in the formation of complex a, I and b (Fig. 3 D). Oligonucleotides corresponding to the immunoglobulin κ B binding site (IgK) and ecdysone receptor binding site (EcR) were used as a negative controls in these competition studies, and revealed no significant elimination of complexes apart from a slight decrease in overall intensity (Fig. 3 D). Binding of complex a was competed by consensus binding sites for all isoforms, with Z3 displaying a lower affinity. Complex b was efficiently competed by the consensus binding sites for all isoforms (with the exception of Z4) when compared with the effect of the nonspecific IgK and EcR competitors. On the other hand, complex I was not affected by any competitor (Fig. 3 D). Binding of 6-h ecdysone-stimulated nuclear extracts to probe D6 resulted in the formation of complexes a and b, but with lower affinity. The binding of three other complexes which migrated in between complex a and b was also apparent. Complex a was efficiently competed by the consensus binding sites for all isoforms (with the exception of Z3) when compared with the competition by the nonspecific competitor IgK; complex b was efficiently competed by the consensus binding sites for all isoforms (with the exception of Z4). One of the intermediate complexes binding to D6 was also competed by the consensus binding site for Z1 and Z2. Because BR-C binding sites are similar to the binding sites of FKH, the results indicate that complexes a and b, which form on the proximal promoter, contain either BR-C proteins or FKH. It was not possible to establish the identity of specific BR-C isoforms that bind *dronc* promoter in this experiment, as all four BR-C isoforms are known to bind to a single BR-C consensus sequence (von Kalm et al., 1994). However, these experiments do suggest that some BR-C specificity exists in the mechanism of *dronc* regulation.

Expression of BR-C transactivates the *dronc* promoter

The finding that BR-C proteins may be interacting with the *dronc* proximal promoter needed to be confirmed using an alternative approach. A luciferase reporter construct containing a 2.75-kb region (from the transcription start site) of the *dronc* promoter was transiently transfected into *l(2)mbn* cells together with expression constructs for different BR-C isoforms. The *dronc* promoter-reporter alone had low basal activity; however, coexpression of the BR-C Z1 isoform resulted in a >40 -fold activation of the reporter expression (Fig. 4 A). Cotransfection of either BR-C Z2 or Z3 had no significant effect (Fig. 4 A). These results indicate that the BR-C Z1 isoform is capable of binding to and transactivating the *dronc* promoter.

Because our binding data revealed that BR-C proteins may possibly be interacting with the D4–D6 probes that corre-

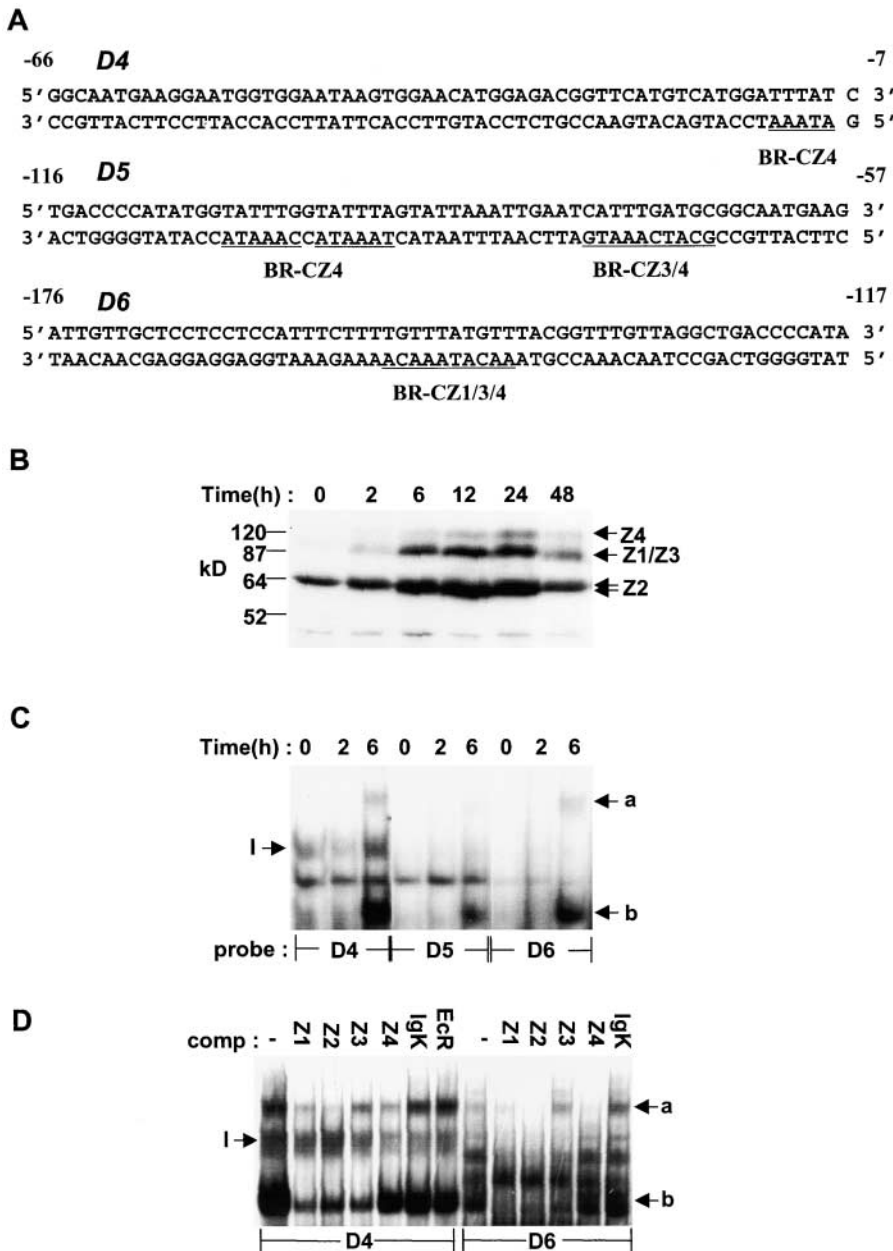


Figure 3. BR-C isoforms bind and regulate *dronc* promoter. (A) Probes used in EMSA are shown. D4 spans from -7 to -66, D5 from -57 to -116, and D6 from -117 to -176. Putative binding sites for BR-C are underlined. (B) Immunoblot analysis of BR-C proteins was performed on extracts from *l(2)mbn* cells treated with 10 μ M ecdysone for the indicated time using BR-C core antibody. The positions of different BR-C isoforms are indicated. (C) Nuclear extracts were prepared from 7.5×10^6 cells treated with 10 μ M ecdysone for 0, 2, or 6 h. 10 μ g of nuclear extract was incubated with D4, D5, and D6 for 20 min, complexes resolved on an acrylamide/TBE gel, dried on 3 MM Whatmann paper and exposed to Kodak film overnight. Ecdysone inducible complexes a, b, and I are indicated. (D) EMSA was performed as in C; however, nuclear extracts were incubated with 40 ng of competitor oligonucleotide corresponding to BR-C isoforms Z1, Z2, Z3, and Z4 or non-specific competitors IgK and EcR. Competition experiments were performed with D4 and D6 probes.

spond to the *dronc* proximal promoter region, we wished to determine whether the proximal promoter region harboring these sites can be transactivated by the Z1 isoform. In addition, we wished to establish whether the proximal promoter is responsible for the Z1-mediated transactivation, or whether there are other Z1 responsive regions present within the *dronc* promoter. Deletions of the *dronc* promoter were subcloned into the luciferase reporter vector ppxG. ppxG alone was not transactivated by the Z1 isoform; however, the reporter containing the 2.75-kb *dronc* promoter was transactivated with a 30-fold induction (Fig. 4 B). Deletion of the promoter to 1.0 kb had no effect, and deletion to 0.52 kb only slightly reduced the fold activation in response to Z1 transactivation. The deletion of the proximal promoter harboring the D4–D6 sequences to -6 bp dramatically reduced Z1-mediated transactivation (Fig. 4 B). These results indicate that the proximal promoter harboring the D4–D6 se-

quences is responsible for most of the transactivation resulting from overexpression of the Z1 isoform.

Induction of *dronc* transcription by ecdysone is mediated by BR-C

To further establish the role of BR-C in ecdysone-mediated *dronc* transcription, we used RNAi to ablate inducible expression of BR-C and tested its effect on *dronc* transcription in response to ecdysone. To confirm the specific elimination of BR-C protein, immunoblot analysis was carried out on *l(2)mbn* cell lysates. All four BR-C isoforms are expressed with ecdysone induction with no effect by the negative control dsRNAi (Fig. 4 C). *BR-C* dsRNAi significantly reduced the expression of all four BR-C isoforms, allowing us to determine the role of BR-C in *dronc* transcription. Transient transfections were carried out with the *dronc* luciferase reporter construct together with *BR-C* RNAi. The *dronc* lu-

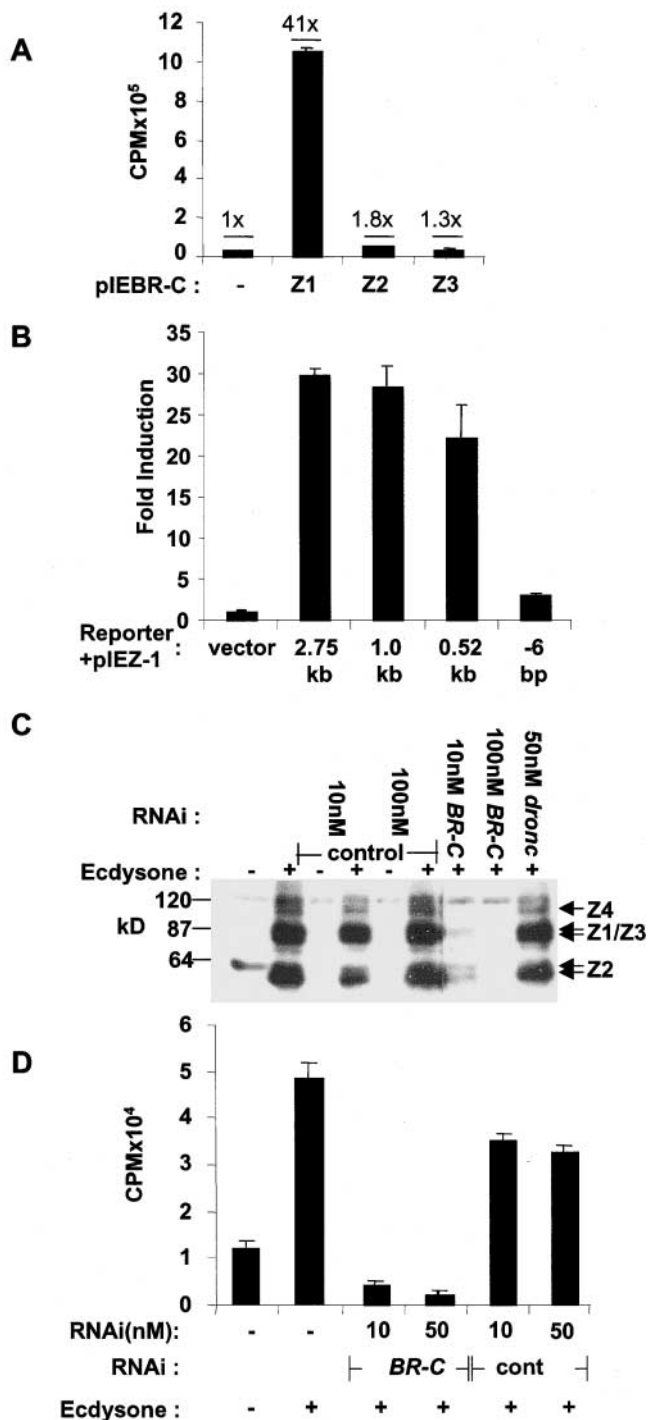


Figure 4. DRONC is regulated by BR-C. (A) 2.5×10^6 *l(2)mbn* cells were transfected in duplicate with $4 \mu\text{g}$ of *dronc* luciferase reporter, pxpDR2.75kbLuc with either $2 \mu\text{g}$ of empty pIE vector (-) or BR-C isoform expression vectors pIEBR-CZ1, Z2, and Z3. Cell extracts were prepared 48 h later and $100 \mu\text{g}$ protein assayed in duplicate for luciferase activity. Fold inductions were calculated by expressing levels of induction as a ratio to levels of reporter alone with pIE empty expression vector. Error bars represent SD. (B) 2.5×10^6 *l(2)mbn* cells were transfected in duplicate with $4 \mu\text{g}$ of luciferase reporter pxpG or *dronc* luciferase reporter, pxpDR2.75kbLuc, pxpDR1.0kbLuc, pxpDR0.52kbLuc, pxpDR6bpLuc alone or with $2 \mu\text{g}$ of expression vector pIEBR-CZ1. Cell extracts were prepared 48 h later and $100 \mu\text{g}$ protein assayed in duplicate for luciferase activity. Fold inductions were calculated by expressing levels of Z1-mediated induction as a ratio to levels of reporter alone and graphed as fold

ciferase reporter construct was induced approximately five-fold by ecdysone treatment. Cotransfection of either 10 or 50 nM of *BR-C* RNAi directed toward all four isoforms greatly reduced transactivation to below basal levels (Fig. 4 D). However, the cotransfection of a negative control RNAi had no significant effect on ecdysone-mediated reporter induction displaying the specificity of the dsRNAi (Fig. 4 D).

Similar experiments were performed on endogenous DRONC. As expected, ecdysone treatment induced expression and processing of DRONC (Fig. 5 A). Transfection of 10 or 100 nM of negative control RNAi had no effect on DRONC expression. However, RNAi to BR-C significantly reduced the expression of *dronc*. The positive control *dronc* RNAi eliminated expression as expected (Fig. 5 A). The results were further confirmed by analysis of *dronc* mRNA. Total RNA extracted from *l(2)mbn* cells showed *dronc* induction in response to ecdysone treatment. The negative control dsRNA had no effect, whereas as little as 10 nM dsRNA to BR-C reduced *dronc* expression (Fig. 5 B). The positive control *dronc* dsRNA eliminated expression as expected (Fig. 5 B). Because ablation of BR-C expression reduces the expression of *dronc* at the level of both RNA and protein, we speculated that BR-C RNAi would also have an effect on ecdysone-induced cell death. Therefore, *l(2)mbn* cells were depleted of BR-C by RNAi, and as a negative control, non-specific RNAi (BP5A) was used. Results show that 70 and 85% of the cells that had no RNAi or nonspecific RNAi had undergone apoptosis in response to ecdysone treatment. In contrast, depletion of BR-C significantly decreased the levels of apoptosis (Fig. 5 C). Although the ablation of BR-C clearly affects the degree of cell death in response to ecdysone, total ablation of cell death was not observed. This is probably because *BR-C* RNAi does not completely abolish DRONC expression, although it does reduce expression of DRONC. Thus, small levels of DRONC may account for the residual apoptosis observed with *BR-C* RNAi. Collectively, these results show that elimination of BR-C expression significantly lowers *dronc* expression in response to ecdysone, therefore displaying that BR-C is crucial for endogenous *dronc* transcription and the overall apoptotic response.

dronc transcript is reduced in *BR-C* mutant flies

Our studies of *l(2)mbn* cells indicate that *BR-C* is essential for proper transcription of *dronc*. If correct, mutations in *BR-C* should impact *dronc* transcription in vivo. Control and *BR-C rbp⁵* mutant animals (Belyaeva et al., 1981) were staged as late third instar larvae, prepupae, and pupae. Total RNA was extracted from these animals and analyzed by Northern blot hybridization with *dronc* and *rp49*. *dronc* transcript was not present at -18 h (relative to pupariation);

induction. Error bars represent SD. (C) 2×10^6 cells were treated with either 10 and 100 nM of control dsRNA (Nedd4) or similar amounts of BR-C dsRNA followed with ecdysone stimulation for 24 h where indicated. $50 \mu\text{g}$ of protein was analyzed by immunoblotting with BR-C core antibody. The position of different BR-C isoforms are indicated. (D) 2.5×10^6 *l(2)mbn* cells were seeded in duplicate and transfected with $4 \mu\text{g}$ of pxpDR2.8kbLuc and allowed to recover for 24 h. Cells were then exposed to dsRNA to *BR-C* (*BR-C*) or negative control (cont) for 24 h followed by $10 \mu\text{M}$ of ecdysone for 24 h. Luciferase assays were performed as in A and B above.

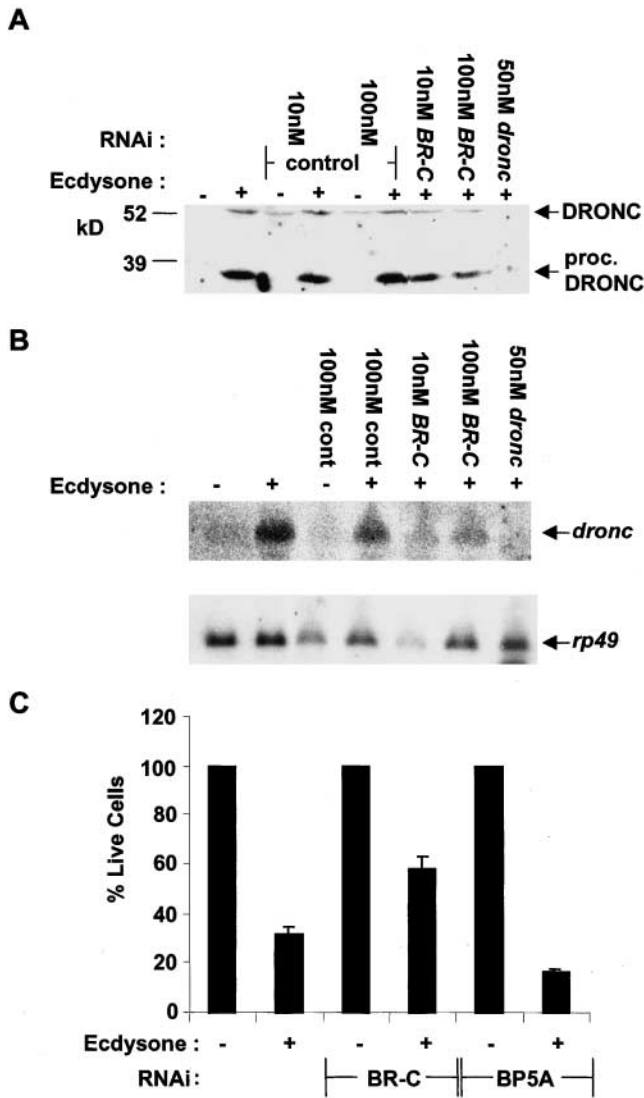


Figure 5. BR-C mediates ecdysone induced *dronc* transcription and cell death. (A) The filter in Fig. 4 C was stripped and blotted with DRONC antibody. Full-length and processed (proc.) DRONC species are indicated. (B) Cells were treated as in Fig. 4 C, and total RNA analyzed by Northern blotting using either a *dronc* or a control *rp49* probe. (C) 2×10^6 cells were treated in duplicate with 37 nM BR-C dsRNA, nonspecific murine N4WBP5A dsRNA (BP5A), or left untreated. Cells were then exposed to 10 μ M ecdysone for 48 h where indicated. Surviving cells were counted by Trypan blue exclusion and percentage live cells were calculated by comparing ecdysone-treated cells with nontreated cells (100%).

however, it is dramatically upregulated from -4 to 4 h, consistent with the induction of *dronc* transcription in response to the late larval ecdysone pulse (Fig. 6). Levels of *dronc* transcript then decrease until 14 h after puparium formation, where it is highly upregulated in response to the ecdysone pulse which triggers salivary gland cell death (Fig. 6). BR-C *rbp5* mutation (Belyaeva et al., 1981) had no effect on the levels of *rp49* transcription (Fig. 6). However, the levels of *dronc* transcript were significantly reduced during this developmental stage in *rbp5* mutant animals (Fig. 6) that are deficient in BR-C Z1 isoform (Emery et al., 1994). These results were further confirmed from the analysis of *dronc* expression in late larvae in BR-C-null *npr1* mutant (unpub-

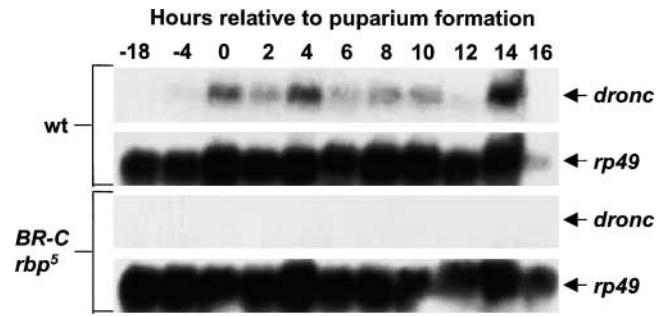


Figure 6. Reduced *dronc* expression in BR-C mutant flies. Total RNA was isolated from wild-type Canton-S (wt) and BR-C (*rbp5*^Y) mutant larvae, prepupae, and pupae staged at 25°C. RNA was analyzed by Northern blotting using *dronc* or *rp49* probes.

lished data). These observations are consistent with BR-C playing a critical role in the regulation of *dronc* transcription and programmed cell death during development.

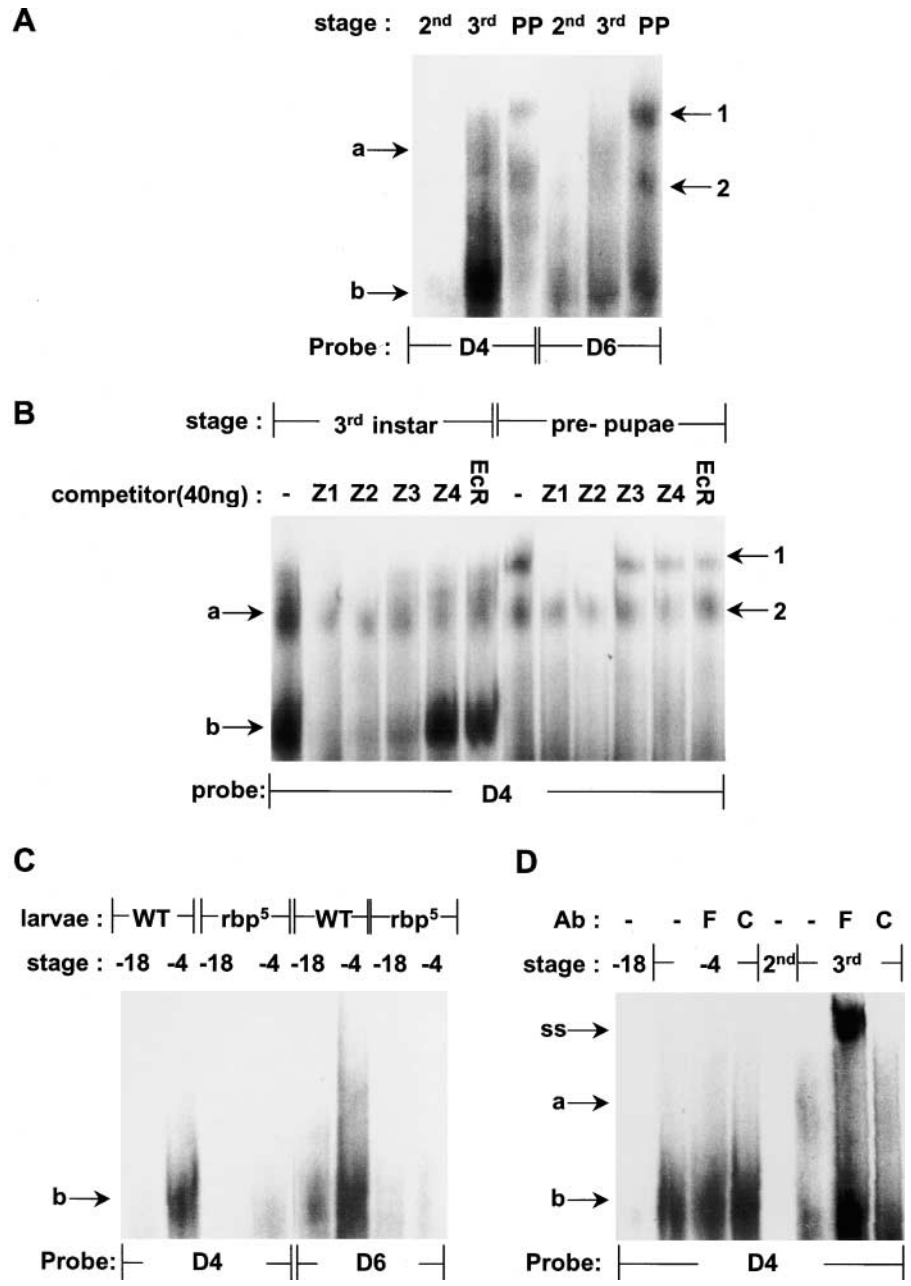
Larval and prepupal nuclear proteins bind *dronc* proximal promoter

Investigation of ecdysone-induced complexes binding to the *dronc* proximal promoter revealed the formation of two complexes (a and b) using nuclear extracts from *l(2)mbn* cells, which were competed by BR-C consensus site competitors (Fig. 3, C and D). These data suggested that either BR-C or FKH bind to the proximal promoter, as they have similar binding sites. As transactivation experiments and RNAi and *rbp5* functional data clearly establish a role for the Z1 isoform in *dronc* transcription, we tested whether similar complexes that were detected in *l(2)mbn* cells are also seen in larvae and prepupae. Nuclear extracts were prepared from larvae at the second instar, wandering third instar, and prepupae. The wandering third instar larval stages from mid third instar until just before puparium formation should contain larvae that express FKH and BR-C proteins (Emery et al., 1994; Mach et al., 1996; Renault et al., 2001). No complexes were observed by EMSA using the D4 probe and second instar larval nuclear extracts (Fig. 7 A). In contrast, a slow- (complex a) and fast-migrating (complex b), complex were observed when extracts from third instar larvae were used in EMSA experiments. These complexes appear to be similar to the complexes observed in *l(2)mbn* cells. Consistent with observations in *l(2)mbn* cells, complex b appeared to bind with a stronger affinity. Extracts from prepupae reveal a decrease in binding of complex b and the formation of two complexes (1 and 2). These complexes have different mobilities than complex a from the third instar stage. The formation of complexes 1 and 2 indicate that prepupal specific proteins bind to these sites and could possibly have a role in mediating *dronc* transcription in response to ecdysone at the prepupal stage. Binding to probe D6 reveals similar binding profiles; however, binding of complex b is not reduced at the prepupal stage (Fig. 7 A).

Because complexes a and b have similar mobility to those from *l(2)mbn* cells, we predicted that they can be competed by oligonucleotides containing BR-C consensus sites. Nuclear extracts from wandering third instar larvae bind complexes a and b using a probe D4 in EMSA experiments (Fig. 7 B). The

Figure 7. **Late larval specific promoter-bound complex is abolished in nuclear extracts from *rbp⁵* flies.**

(A) Nuclear extracts were prepared from second instar, wandering third instar larvae and prepupae. 10 μ g of nuclear extract was incubated with *dronc* probes D4 and D6 for 20 min, complexes resolved on a acrylamide/TBE gel, dried on 3 MM Whatmann paper, and exposed to Kodak film overnight. Inducible complexes a, b, 1, and 2 are indicated. (B) 10 μ g of nuclear extracts from wandering third instar larvae or prepupae were incubated with probe D4 alone or with 40 ng of cold BR-C isoform consensus competitors or nonspecific EcR competitor. Third instar larval complexes a and b and prepupal complexes 1 and 2 are indicated. (C) 10 μ g of nuclear extracts prepared from wild-type and *rbp⁵* larvae specifically staged at -18 and -4 h (relative to puparium formation) were incubated with probes D4 and D6. Complex b is indicated. (D) 10 μ g of nuclear extracts prepared from wild-type larvae from second instar, wandering third instar or specifically staged at -18 h, -4 h (relative to puparium formation) were incubated with probe D4. Where indicated, 2 μ l of FKH antibody (F) or a rabbit nonspecific control antibody (C) was added to the extracts before the addition of probe.



nonspecific competitor EcR had no effect on complexes, but a slight decrease in overall intensity was evident. Binding of complex a is decreased with the Z1 and Z2 competitors when compared to the effect of the EcR competitor. Binding of complex b is competed by Z1, Z2, and Z3. Complex 1 in prepupal extracts is competed by Z1 and Z2, whereas EcR has no effect (Fig. 7 B). The same results were also seen for probe D6 (unpublished data). These results indicate that similar complexes isolated from nuclei of wandering third instar and *l(2)mbn* cells bind to the proximal promoter of *dronc*, and could possibly contain either BR-C or FKH.

Loss of BR-C Z1 in flies abolishes binding of the ecdysone-inducible complex to *dronc* proximal promoter

Previous studies indicate that BR-C Z1 protein expression is very low at 18 h (-18) prior to pupariation, and increases significantly by 4 h (-4) prior to pupariation (Emery et al.,

1994). These results are also in agreement with the transcript profiles of the Z1 isoform (Huet et al., 1993). Because *dronc* is highly transcribed in response to the late larval ecdysone pulse and is expressed from -4 to 4 h relative to puparium formation (Fig. 6), we examined whether nuclear complexes bind to proximal promoter at this precise developmental stage. In addition, we tested whether nuclear extracts from *BR-C rbp⁵* larvae, which are deficient in expression of the BR-C Z1 isoform (Emery et al., 1994), exhibit decreased formation of complexes. EMSA experiments showed that at -18 h, there are no nuclear complexes binding to the D4 probe (Fig. 7 C). In contrast, a single complex, which migrates similarly to complex b from wandering third instar larvae, binds strongly at the -4-h developmental stage (binding of complex a was not evident). Analyses of *rbp⁵* mutants from the same developmental stages show that complex b is no longer present in extracts where the Z1 protein is no

longer expressed (Fig. 7 C). A faint diffuse complex is observed; however, its migration is slightly faster and could possibly represent a complex which was previously masked by the complex b. Binding to the D6 probe was identical (Fig. 7 C). These results indicate that at the time when *dronc* is expressed (−4 h), only complex b binds to the proximal promoter, and that this complex contains the Z1 protein.

However, it is possible that Z1 can induce expression of another transcription factor that binds to these probes. To test whether complex b contains FKH, we used an FKH antibody that has previously been shown to recognize FKH in EMSA experiments (Mach et al., 1996). Nuclear extracts from −18-h larvae revealed no complexes binding to the D4 probe (Fig. 7 D). At −4 h, complex b binds strongly to the probe, and addition of FKH antibody has no effect on the complex (Fig. 7 D). Treatment of extracts with the same amount of nonspecific rabbit antibody also had no effect.

Because complex a binds to the proximal promoter but is only present in nuclear extracts from wandering third instar larvae, we wished to determine its identity. Previous work has shown that FKH is highly expressed in response to the small ecdysone pulse at the middle of the third instar stage (ecdysone pulse that triggers wandering behavior), and that the levels drop as expression is suppressed in response to the late larval ecdysone pulse (Renault et al., 2001). Therefore, it is possible that this complex could contain FKH. No complexes bound to the D4 probe with nuclear extracts from second instar larvae, whereas the appearance of both complex a and b was evident from wandering third instar larvae (Fig. 7 D). The addition of FKH antibody not only specifically eliminated binding of complex a, but also resulted in a supershift illustrating that complex a contains FKH (Fig. 7 D). Complex b was not abolished and possibly increased in intensity. The addition of an equal amount of a control antibody had no significant effect, demonstrating its specificity (Fig. 7 D). In summary, complex a contains FKH and binds to the promoter possibly at the mid third instar stage when FKH is highly expressed. Complex b binds to the proximal promoter specifically at −4 h relative to pupation when *dronc* is transcribed and consists of Z1 (or a Z1-induced protein).

Discussion

The data presented in this paper show for the first time that ecdysone-mediated *dronc* transcription is regulated by BR-C, and that this upregulation of *dronc* is essential for ecdysone-induced cell death. The effect of BR-C RNAi on the endogenous *dronc* gene in *l(2)mbn* cells indicates that BR-C proteins are important for *dronc* transcription. Transactivation experiments using the *dronc* promoter linked to a reporter gene indicate that the BR-C Z1 isoform in particular is likely to be an important regulator of ecdysone-mediated *dronc* transcription. A lack of *dronc* expression in larvae, prepupae, and pupae from BR-C *rbp5* mutants further supports BR-C Z1 playing a key role in *dronc* transcription. These data are also supported by the earlier observation that larval salivary gland cell death is defective in BR-C *rbp5* mutants (Jiang et al., 2000). Although the data presented in this paper clearly indicate that BR-C plays a crucial role in the regulation of *dronc* transcription, there were clear differences

between the *l(2)mbn* results as opposed to data from larval/prepupal stages. For example, larvae and prepupae from *rbp5* mutants lack any detectable *dronc* transcript, whereas in *l(2)mbn* cells, RNAi-mediated BR-C ablation does not completely inhibit *dronc* expression or ecdysone-induced cell death. These differences are likely to be due to different transcriptional regulatory mechanisms in hemocyte-derived *l(2)mbn* cells and in larvae/prepupae in vivo.

The role of BR-C proteins has been extensively studied because of their role in the regulation of the salivary gland secretory proteins (*sgs*). 19 BR-C binding sites were found in the *sgs-4* promoter, and 13 of them were bound by more than one isoform (von Kalm et al., 1994). The high variability of these AT rich sequences, and the need for high amounts of recombinant protein to detect binding, suggests that other proteins and/or flanking sequences are needed for BR-C binding. Although BR-C mutant flies display no *sgs-4* transcription, experiments failed to detect binding of BR-C proteins to the *sgs-4* promoter. Thus, BR-C proteins may activate the *sgs-4* promoter via an indirect mechanism, or possibly bind to the promoter in a transient nature (Renault et al., 2001). Furthermore, FKH has been found to bind to the BR-C sites using nuclear extracts (Lehman and Korge, 1996). FKH may control *sgs-4* transcription through BR-C sites, as mutations of these sites leads to reduced expression of a reporter gene (von Kalm et al., 1994). The *sgs-4* gene is highly expressed at the mid third instar larval stage when FKH is also expressed, and is repressed in response to the late larval ecdysone pulse where FKH expression is reduced (Renault et al., 2001). In contrast, *dronc* is not expressed in mid third instar larvae, but is highly induced in response to the late larval ecdysone pulse and again 12 h after pupation. Thus, *dronc* transcription is similar to the expression pattern of the BR-C Z1 isoform. However, we have found that at the exact stage of development when *dronc* is induced, FKH binding is not detected at the proximal promoter. In contrast, we detect a complex that binds to the *dronc* promoter at −4 h relative to pupariation, which is eliminated in larvae which do not express Z1. Our attempts to use BR-C antibodies in EMSA to supershift the complex have failed for both nuclear extracts and also recombinant proteins, hence questioning the ability of the antibodies to supershift the BR-C proteins.

To our knowledge, this is the first time it has been shown that binding of a nuclear complex to a promoter region containing BR-C binding sites is abolished in Z1-deficient larvae. Our transactivation data also demonstrate for the first time that the Z1 isoform can in fact bind to the *dronc* promoter and activate its expression in transient transfections, and most of the Z1-mediated activity is localized to the proximal promoter containing the BR-C binding sites. Future experiments will focus on the role of FKH in *dronc* transcription, its exact stage of binding to the *dronc* promoter, analysis of other proteins present in the ecdysone-induced complexes, and mutational analysis of the *dronc* promoter in transgenic flies. Overall, our data presented in this paper are consistent with a model of developmental cell death where specific upstream signals can directly lead to the upregulation and activation of death effector machinery via specific temporally and spatially controlled transcription factors.

Materials and methods

Cells and caspase assays

l(2)mbn cells, a gift from Dr. A. Dorn (Johannes Gutenberg University, Mainz, Germany) (Ress et al., 2000) were grown in Schneider's medium supplemented with 10% FBS. Cells, 1.5×10^6 /well, were seeded in 6-well plates in duplicate. Where necessary, ecdysone (10 μ M; Sigma-Aldrich) was added for desired time. Cells' viability was assessed by Trypan blue exclusion. Cells lysates were prepared by freeze thawing and clarified by centrifugation at 13 K rpm for 5 min. 50 μ g of lysate was assayed for caspase activity using VDVAD-amc and DEVD-amc substrates as previously described (Dorstyn et al., 1999b; Harvey et al., 2001).

Cell death assays

Cell death assays in transfected *l(2)mbn* cells were carried out essentially as described previously (Colussi et al., 2000; Doumanis et al., 2001).

Northern blotting

Total RNA was extracted from *l(2)mbn* cells using Trizol reagent (Life Technologies) and ~ 20 μ g was analyzed by Northern blotting using 32 P-labelled *dronc* or *rp49* probes as previously described (Dorstyn et al., 1999a, 1999b; Colussi et al., 2000). For analysis of *dronc* transcription in BR-C mutants, total RNA was isolated from Canton S wild-type control and BR-C (*rbp⁵N*) mutant whole animals that were staged in hours relative to puparium formation at 25°C. Larvae, prepupae, and pupae were staged, RNA extracted, and analyzed by Northern blotting as previously described (Baehrecke and Thummel, 1995; Fletcher and Thummel, 1995). Control and mutant Northern blots were cohybridized with probes to detect *dronc* and *rp49* as a loading and transfer control.

Immunoblotting

Cell lysates were electrophoresed on a 10% SDS PAGE, transferred onto PVDF membrane (Schleicher & Schuell), and blocked for 4 h in 5% skim milk. Affinity-purified anti-DRONC antibody (Quinn et al., 2000) was used at a 1:300 dilution; BR-C core antibody, a gift from Dr. Jean-Antoine Lepesant (Institute Jaques Monod, Paris, France) was used at a 1:1,000 dilution; and secondary HRP-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech) was used at 1:2,000 dilution. Signals were detected using ECL system (Amersham Pharmacia Biotech). A cytochrome C antibody was purchased from Pharmingen and used at a 1:2,000 dilution as described (Dorstyn et al., 2002).

Expression constructs

A luciferase reporter ppxGDR2.75kbLuc was constructed by Dr. P. Colussi (Hanson Center for Cancer Research, Adelaide, Australia). This construct contains a 2.75-kb region of *dronc* promoter upstream of transcription start site up to the ATG cloned into the luciferase reporter vector, ppxpG, provided by Dr. P. Cockerill (Hanson Center for Cancer Research). Deletions of the promoter were generated by PCR amplification from 1.0 kb, 0.52 kb, and -6 bp, relative to transcription start site up to the ATG and cloned into the ppxpG luciferase reporter vector. Bacterial expression constructs for the BR-C isoforms were provided by Dr. L. von Kalm (University of California, Berkeley, CA), and included BR-CcoreQ1-Z1 in the pET-FM vector and Q1-Z1 and NS-Z3 in the pDS-MCS expression vectors (von Kalm et al., 1994). BR-C isoforms were amplified using the bacterial expression constructs as templates and Pfu turbo and cloned into the Pme I and Bgl II sites of the insect expression vector, pE1-4 (Novagen).

RNA interference

Regions of cDNA for *dronc* (nucleotides 781–1047), BR-C (nucleotides 392–1060), and the negative control mouse Nedd4 (190 bp from the WW1 coding region) (Kumar et al., 1997) or N4WBP5A (~ 700 bp coding region) (Harvey et al., 2002), were PCR amplified using appropriate primers and cloned into pGEM-T Easy (Promega). Plasmids were linearized and RNA synthesized using T7 and SP6 Megascript kits (Ambion). Sense and antisense strands were annealed to generate dsRNA and quality of RNA analyzed on agarose gel. dsRNA (10–100 nM) was added to cells in 1 ml serum-free media and mixed vigorously. Cells were incubated for 1 h followed by the addition of 2 ml of media supplemented with 10% FBS. Cells were incubated overnight and then treated with ecdysone.

Preparation of nuclear extracts and EMSA

7.5×10^6 *l(2)mbn* cells were pelleted, washed once in PBS, and resuspended in 800 μ l of buffer A (10 mM Hepes, pH 7.6, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM EGTA, Complete™ protease inhibitors; Roche), and placed on ice for 15 min. NP40 was added to 0.1%, vortexed

for 30 s, and centrifuged at 13 K for 30 s at 4°C. Nuclear pellet was resuspended in 80 μ l buffer C (10mM Hepes, pH 7.6, 400 mM NaCl, 7.5 mM MgCl₂, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, Complete™), and incubated on ice for 40 min while shaking. Extract was then centrifuged for 5 min at 13 K and supernatant aliquoted and frozen at -70° C. Nuclear extracts from second instar, wandering third instar, early pupae, and larvae specifically staged at -18 and -4 h (relative to puparium formation) from wild-type and *rbp⁵* larvae were prepared as follows. Extracts were made by homogenizing 50 larvae in 300 μ l of buffer A and removing them to a fresh tube in a total of 600 μ l devoid of larval debris. After incubation on ice for 15 min, 10 μ l of rabbit FKH antibody, provided by Dr. Václav Mach (Institute of Entomology Czech Academy of Sciences Brno, Czech Republic) or nonspecific rabbit antibody was also added to the nuclear extracts and incubated on ice before the addition of labelled probe. In competition experiments, 40 ng of cold competitor oligonucleotide was also included. 0.2 ng of labelled probe was added and incubated on ice for a further 20 min. 5 μ l of 5 \times acrylamide loading buffer was added, and the reaction run on a 5% polyacrylamide/0.5 \times TBE gel. The gel was then dried down onto 3 MM Whatmann paper and exposed to Kodak x-ray film.

Transfections/luciferase assay

2 μ g of ppxGDR2.75kbLuc, ppxGDR1.0kbLuc, ppxGDR0.52 kbLuc, or ppxGDR-6bpLuc luciferase construct, was transfected alone or with 2 μ g of BR-C expression constructs. Equal amounts of DNA were used with the pE1-4 expression vector and Cellfectin. DNA in a total volume of 100 μ l in Schneider media (without FBS) was added to Cellfectin (2:9) in 100 μ l total media devoid of FBS and incubated at room temperature for 15 min. 800 μ l of serum-free media was added and then overlaid onto 2.5×10^6 cells in 6-well plates. Cells were incubated with the DNA/Cellfectin mixture for 5 h. The medium was replaced by 3 ml of Schneider media supplemented with 10% FBS and cells allowed to recover for 24 h. Where needed, ecdysone (10 μ M) was added for 24 h. Cells were harvested 48 h posttransfection, resuspended in lysis buffer (100 mM phosphate buffer, pH 7.8, 10 μ M EDTA, 2 mM DTT), and frozen three times in liquid nitrogen. After centrifugation for 5 min at 13 K, supernatant was analyzed for luciferase activity. Protein (60–100 μ g) was assayed in 200 μ l assay buffer (100 mM phosphate buffer, 8 mM MgSO₄, 2 mM DTT, 0.75 mM ATP, 0.175 mM coenzyme A) using an illuminometer (Packard).

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