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Ecdysone receptor directly binds the promoter of the *Drosophila* caspase *dronc*, regulating its expression in specific tissues

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The steroid hormone ecdysone regulates moulting, cell death, and differentiation during insect development. Ecdysone mediates its biological effects by either direct activation of gene transcription after binding to its receptor EcR–Usp or via hierarchical transcriptional regulation of several primary transcription factors. In turn, these transcription factors regulate the expression of several downstream genes responsible for specific biological outcomes. DRONC, the *Drosophila* initiator caspase, is transcriptionally regulated by ecdysone during development. We demonstrate here that the *dronc* promoter directly binds EcR–Usp. We further show that mutation of the EcR–Usp binding element (EcRBE) reduces transcription of a reporter and abolishes transactivation by an EcR isoform. We demonstrate that EcRBE is required for temporal regulation of *dronc* expression in response to ecdysone in specific tissues. We also uncover the participation of a putative repressor whose function appears to be coupled with EcR–Usp. These results indicate that direct binding of EcR–Usp is crucial for controlling the timing of *dronc* expression in specific tissues.

Introduction

Programmed cell death (PCD) is an essential biological process required for the sculpturing of various tissues and removal of unwanted cells during development. PCD is mainly executed by the process of apoptosis and involves a highly conserved machinery (for review see Baehrecke, 2002; Adams, 2003). Various signals such as cytotoxic insults, hormones, and growth factors regulate the activation of PCD by controlling the balance between pro- and anti-death factors of the cell death machinery (Baehrecke, 2002; Adams, 2003). 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 recent studies suggest that transcription plays a key role in the control of the cell death machinery by regulating the intracellular levels of the pro- and anti-death factors (for review see Kumar and Cakouros, 2004).

In *Drosophila melanogaster* a single steroid hormone 20-hydroxyecdysone (ecdysone) regulates PCD to remove obsolete larval tissues (for review see Riddiford, 1993; Thummel, 1996; Baehrecke, 2000, 2002; Truman and Riddiford, 2002). 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. An ecdysone pulse toward the end of the larval stage signals puparium formation and histolysis of the larval midgut. A second pulse ~12 h later initiates head eversion and histolysis of the larval salivary glands. These events are followed by progenitor cells giving rise to adult tissues (Thummel, 1996; Baehrecke, 2000, 2002; Truman and Riddiford, 2002). Ecdysone binds to its heterodimeric receptor, EcR–Usp (ecdysone receptor–ultraspiracle), and transcriptionally regulates several primary response genes. There are three EcR isoforms in *Drosophila*, EcR-A, B1, and B2 (Yao et al., 1993). These isoforms are highly homologous in the DNA and ligand binding domains but differ in their amino terminal transactivation domain. The EcR-B1 isoform is predominantly expressed in tissues destined to undergo PCD, whereas the EcR-A isoform is expressed in tissues that differentiate in response to ecdysone (Talbot et al., 1993; Yao et al., 1993).

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Abbreviations used in this paper: EcRBE, EcR–Usp binding element; EMSA, electrophoretic mobility shift analysis; PCD, programmed cell death; TSA, Trichostatin A.

In the larval salivary glands and midgut, ecdysone controls the expression of several transcription factors, which in turn regulate several secondary response genes (Jiang et al., 1997; Baehrecke, 2000, 2002). EcR–Usp and ecdysone-induced transcription factors βFTZ-F1, BR-C, E74, E75, and E93 have been shown to play a role in ecdysone-mediated cell death in larval salivary gland and midgut. For example, EcR–Usp directly regulates *rpr* transcription in salivary glands and BR-C is required for maximal *rpr* and *dronc* expression (Jiang et al., 2000, Cakouros et al., 2002). BR-C and E74A are also required for the optimal induction of *hid* in salivary glands (Jiang et al., 2000). In salivary glands of the *E93* mutants *rpr*, *hid*, *ark*, and *dronc* mRNA levels are severely reduced (Lee et al., 2000). These results suggest that ecdysone-mediated up-regulation of death initiators such as *rpr*, *hid*, *dark*, and *dronc* is crucial for PCD in salivary glands and midgut during *Drosophila* metamorphosis.

Of the seven caspases in *Drosophila,* DRONC is the CED3/caspase-9-like apical caspase (Dorstyn et al., 1999a; for review see Kumar and Doumanis, 2000; Richardson and Kumar, 2002). DRONC is the only fly caspase containing a caspase recruitment domain, and its function is essential for PCD in *Drosophila* (Hawkins et al., 2000; Meier et al., 2000; Quinn et al., 2000; Dorstyn et al., 2002). Interestingly, *dronc* is transcriptionally regulated by ecdysone in salivary glands and midgut during larval-pupal metamorphosis, and *dronc* overexpression is sufficient to mediate ecdysone-induced PCD (Dorstyn et al., 1999a,b; Cakouros et al., 2002). These studies underscore the importance of direct regulation of caspase levels in initiating PCD in vivo. Furthermore, they provide evidence that developmental PCD is controlled at the level of transcription, rather than activation, of a preexisting cell death machinery alone. Given this evidence, it is essential to understand the transcriptional control of the caspase activation machinery. As ecdysone-mediated *dronc* regulation in *Drosophila* provides a convenient model to study transcriptional regulation of the core PCD machinery we have been dissecting out the *dronc* promoter for important transcriptional regulatory elements. Previous work has shown that BR-C and E93 are important for *dronc* expression by ecdysone (Lee et al., 2000; Cakouros et al., 2002). Preliminary promoter deletion studies suggest that *dronc* transcriptional regulation is complex and involves both temporal and spatial control (Daish et al., 2003). In the present paper, we demonstrate that EcR-B1 directly binds and transactivates the *dronc* promoter and that this binding is necessary for the correct timing of expression of *dronc* in specific tissues. These studies provide a basis for investigating both temporal and spatial regulation of gene transcription of a caspase during development by a steroid hormone receptor.

Results

A specific region of the *dronc* promoter is essential for ecdysone-mediated transcription

In an effort to identify regions of the *dronc* promoter that are essential for ecdysone-mediated transcription, a series of deletion constructs containing 2.8, 1.1, and 0.54 kb of the *dronc* promoter, cloned in front of the luciferase reporter gene, were generated and analyzed for their ability to drive reporter expression by ecdysone. The *Drosophila* cell line *l(2)mbn*, which undergoes ecdysone-induced and *dronc*-dependent cell death, was used in this paper (Ress et al., 2000; Cakouros et al., 2002). Transient transfections with the reporter constructs showed that the 2.8-kb promoter was highly responsive to ecdysone, whereas 1.1 or 0.54 kb promoters were unable to drive reporter expression after ecdysone treatment (Fig. 1 A). These experiments also revealed that deletion of the 2.8-kb

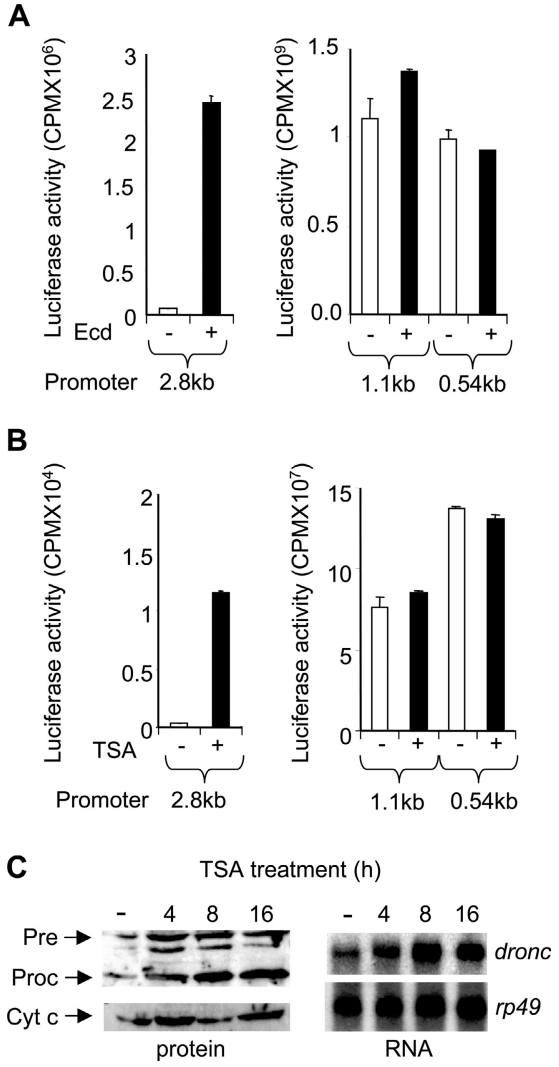

Figure 1. **Upstream *dronc* promoter is responsive to ecdysone and TSA.** (A) 2.5×10^6 *l(2)mbn* cells were transfected in triplicate with 2 μg of *dronc* luciferase reporter, pxpDR2.8kbLuc, pxpDR1.1kbLuc, or pxpDR0.54kbLuc. After 24 h, cells were treated with 10 μM ecdysone (Ecd) for 24 h where indicated (+). Cell extracts were prepared and assayed in triplicate for luciferase activity. Background luciferase activity obtained from empty luciferase vector transfections was subtracted from values shown. Error bars represent SD. (B) Experiment was conducted as in A except trichostatin A (TSA) was used at 1 μM where indicated (+). (C) *l(2)mbn* cells were treated with ethanol for 16 h (−) or with TSA (1 μM) for the indicated time. All cells were harvested at the same time. 10^7 cells were used for immunoblotting using a DRONC antibody. Full-length precursor (Pre) and processed (Proc) DRONC species are indicated. For Northern blot analysis, 15 μg of total RNA was electrophoresed, transferred onto nitrocellulose membrane, and probed with *dronc* probe or a control *rp49* probe.

promoter to 1.1 kb resulted in a dramatic increase in basal promoter activity, suggesting the possible recruitment of a repressor to this region (Fig. 1 A). Given that most repressors recruit histone deacetylases to repress transcription, we used the *dronc* promoter-reporter constructs in transient transfections to determine if they were responsive to the histone deacetylase inhibitor Trichostatin A (TSA). TSA treatment of *l(2)mbn* cells transfected with the *dronc*-reporter constructs revealed that the 2.8-kb promoter was activated by TSA, whereas 1.1 and 0.54 kb promoters had lost the ability to respond to TSA (Fig. 1 B). To further test that *dronc* repression can be alleviated by TSA, we analyzed the endogenous *dronc* transcript and protein levels (Fig. 1 C). In *l(2)mbn* cells treated with TSA for 0–16 h, endogenous DRONC precursor increased at 4 h, and then quickly stabilized as it was processed to its active form (Fig. 1 C). It should be noted that TSA treatment of *l(2)mbn* cells for 16 h results in some apoptosis. Northern blot analysis showed that TSA treatment increased the levels of *dronc* up to 8 h, which then stabilized (Fig. 1 C). These data suggest that alleviation of repression is required for *dronc* expression. Overall, these experiments demonstrate that the region between 2.8 and 1.1 kb of the *dronc* promoter is essential for ecdysone-induced *dronc* transcription and that this region also harbors a putative repressor element, which presumably acts by recruiting a histone deacetylase.

Cycloheximide partially inhibits *dronc* transcription

Although *dronc* has been shown to be regulated by the ecdysone-induced transcription factors BR-C and E93 (Lee et al., 2000; Cakouros et al., 2002), two observations suggest the possibility of EcR–Usp directly binding and activating the *dronc* upstream promoter. First, previous work has shown that ecdysone can regulate *dronc* transcription in *l(2)mbn* very early (~2 h), whereas other transcription factors such as BR-C bind to the promoter after 6 h of ecdysone exposure (Cakouros et al., 2002). Second, the upstream promoter region seems to harbor sites for both repressors and activators (Daish et al., 2003). Nuclear hormone receptors tend to recruit corepressors in the absence of ligand to repress transcription and coactivators in the presence of ligand to activate transcription (Kumar and Thompson, 2003). To examine this possibility, *l(2)mbn* cells were treated with ecdysone for various times in the presence or absence of the protein translation inhibitor cycloheximide. If ecdysone-induced transcription factors are solely responsible for *dronc* transcription, then cycloheximide should inhibit the ecdysone-mediated *dronc* increase. RT-PCR analysis showed that *dronc* transcript levels increased with ecdysone treatment from 0–12 h (Fig. 2 A). In the presence of cycloheximide, *dronc* levels at 0 and 6 h were unaffected but were reduced at 12 h (Fig. 2 A). These results were confirmed by Northern blotting (Fig. 2 B) using earlier time points to demonstrate the early onset of *dronc* transcription, which is insensitive to cycloheximide treatment. Under these conditions, the control *rp49* levels were not significantly affected. These data support the possibility that the *dronc* promoter is directly activated by the preexisting ecdysone receptor, which acts in tandem with other ecdysone responsive transcription factors to activate *dronc* transcription.

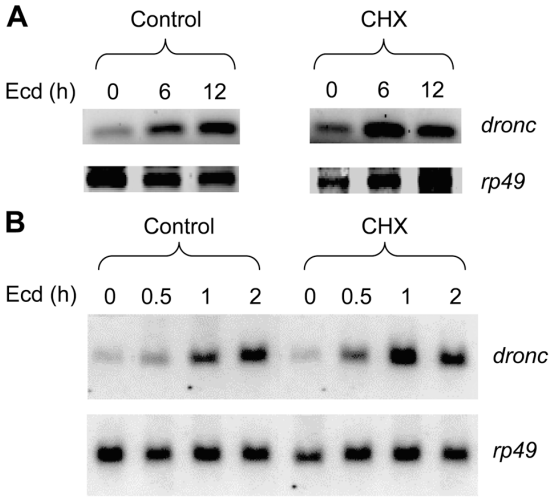

Figure 2. **Ecdysone-mediated *dronc* transcription is partially cycloheximide sensitive.** (A) 10^7 *l(2)mbn* cells were treated with 10 μM ecdysone (Ecd) for the indicated time in the presence or absence (Control) of 10 μg/ml cycloheximide (CHX). RNA extracted from cells was analyzed by RT-PCR. (B) Northern blot analysis was performed on RNA samples from cells treated as in A. Where indicated, cells were treated with cycloheximide (CHX) for 2 h.

dronc promoter contains an EcR–Usp binding element (EcRBE)

To screen for possible binding of EcR–Usp to the *dronc* promoter, we performed electrophoretic mobility shift analysis (EMSA) and competition experiments. EMSA experiments were performed using the *hsp70* EcRBE as a probe and in vitro translated EcR–Usp proteins (Fig. 3 A). The EcR–Usp complex was completely abolished when competed with the cold *hsp*EcRBE oligonucleotide, illustrating the specificity of the complex. 400 bp PCR products spanning the upstream *dronc* promoter region were used as competitors in an attempt to locate the EcRBE. Competitors spanning the region between 2.8 to 1.42 kb had no significant effect on the EcR–Usp complex, however the region between 1.42–1.0 kb clearly competed out most of the EcR–Usp complex (Fig. 3 A). Further experiments mapped the potential EcRBE to the region between 1.42 to 1.2 kb (Fig. 3 B). The ~200-bp region was analyzed in more detail using 60 bp overlapping oligonucleotides spanning the region. As shown in Fig. 3 C, only oligonucleotide 2 successfully competed out the EcR–Usp complex in EMSA, suggesting that the potential EcRBE in the *dronc* promoter resides within this sequence.

An EcR–Usp binding site in the *dronc* promoter

Analysis of the 60 bp region revealed a potential EcR–Usp binding site that has a 10 out of 13 bp match to the consensus EcRBE (Fig. 4 A). An oligonucleotide containing this potential EcRBE was used as a competitor as well as a mutant oligonucleotide that had specific mutations in the binding site (Fig. 4 A). As shown in Fig. 4 B, the EcR–Usp complex with the *hsp*EcRBE could be competed out with the *hsp*EcRBE. Increasing amounts of the *dronc*EcRBE oligonucleotide also competed the EcR–Usp complex, however higher amounts were needed to abolish binding of EcR–Usp

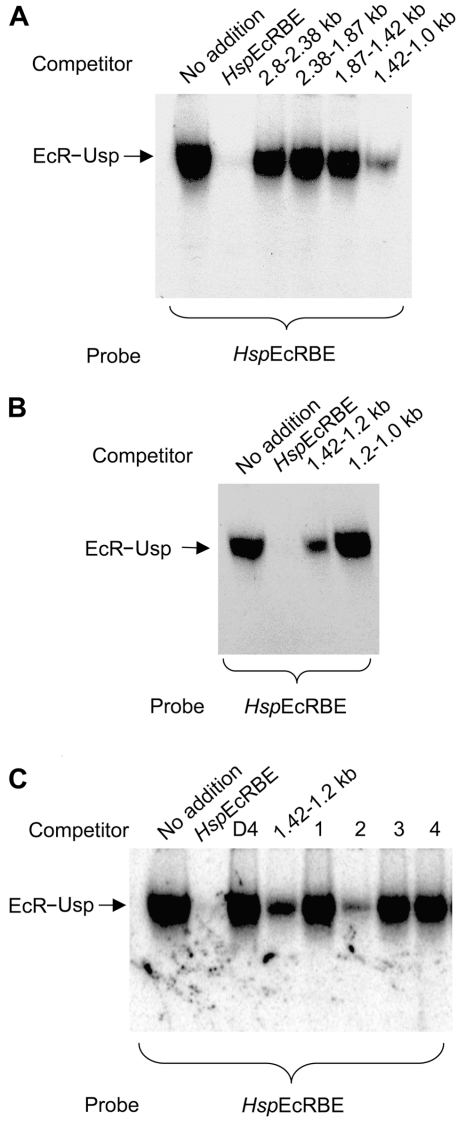

Figure 3. ***dronc* upstream promoter harbors a putative EcRBE.** (A) In vitro translated EcR and Usp proteins were incubated with the EcR consensus probe hspEcRBE for 20 min in the presence of *dronc* promoter fragments. 400 bp PCR fragments spanning the *dronc* promoter region from 2.8 to 1.1 kb were gel purified and 400 ng was used in each reaction. Positive control (*hsp*EcRBE) was used at 40 ng (equimolar). Complexes were resolved on an acrylamide/TBE gel, dried on 3 MM Whatmann paper, and exposed to Kodak film overnight. EcR–UsP complex is indicated. (B) EMSA experiment was performed as in A except PCR fragments used as competitors spanned the regions from 1.42 to 1.0 kb. hspEcRBE was used as the probe. (C) EMSA was performed as in A. Negative control competitor (D4) corresponds to the *dronc* promoter region between 67 to 7 bp upstream of the transcription start site. Oligonucleotide competitors correspond to the *dronc* promoter region 1.44 to 1.2 kb upstream of the transcription start site. *Hsp*EcRBE was used as the probe.

to *hsp*EcRBE. The mutant oligonucleotide failed to compete for the EcRBE (Fig. 4 B). The ability of the *dronc*EcRBE to bind EcR–Usp was determined by EMSA, and as shown in Fig. 4 C, EcR–Usp formed a specific complex that was abolished when competed with the *hsp*EcRBE oligonucleotide. Mutation of the *dronc*EcRBE rendered it incapable of binding EcR–Usp (Fig. 4 C).

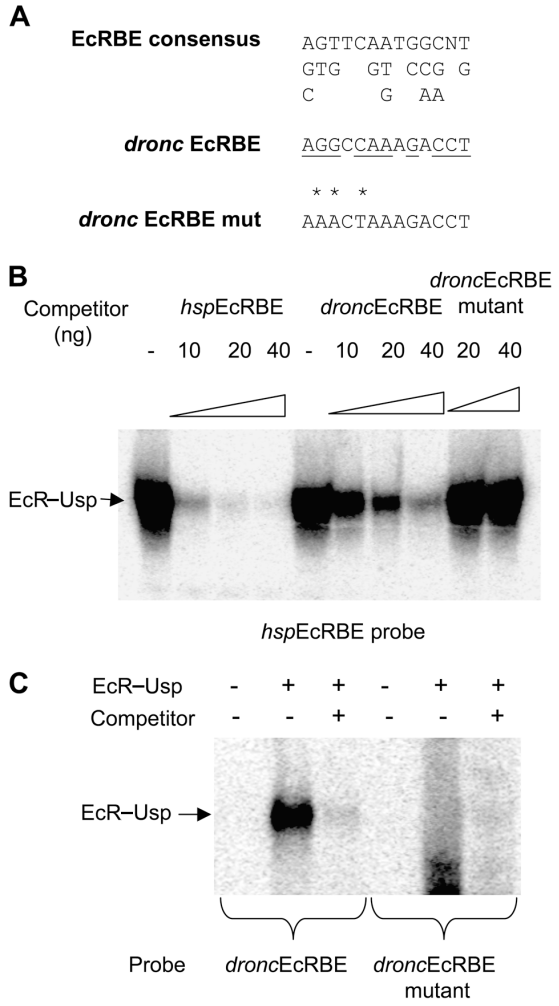

Figure 4. ***dronc*EcRBE binds EcR–Usp.** (A) The EcR consensus binding site, its variations, and the putative *dronc*EcRBE sequences are shown. The conserved residues in the *dronc*EcRBE are underlined. The asterisks represent the residues that have been mutated in a mutant *dronc*EcRBE, which was used in the following studies. These sequences correspond to the oligonucleotides used in EMSA. (B) In vitro translated EcR and Usp proteins were incubated with the EcR consensus probe (hspEcRBE) for 20 min in the presence of increasing amounts of *hsp*EcRBE competitor, *dronc*EcRBE competitor, or *dronc*EcRBE mutant competitor oligonucleotides. Complexes were resolved on an acrylamide/TBE gel, dried on 3 MM Whatmann paper, and exposed to Kodak film overnight. EcR–UsP complex is indicated. (C) EMSA was performed as in B. Probes used were the *dronc*EcRBE and *dronc*EcRBE mutant oligonucleotides.

The EcR-B1 isoform specifically binds to the *dronc* promoter

In *Drosophila*, the EcR-B1 isoform is predominantly expressed in tissues that are destined to undergo PCD, whereas the EcR-A isoform is predominant in tissues which undergo morphogenesis and form adult structures (Talbot et al., 1993). Based on these observations, we predicted that *dronc*, which is expressed in tissues undergoing PCD, is likely to be regulated by the EcR-B1 isoform. Therefore, we analyzed the expression of EcR isoforms in *l(2)mbn* cells that also undergo PCD in response to ecdysone. Using primers specific for each isoform in RT-PCR analysis, we found that the EcR-B1 and EcR-B2 iso-

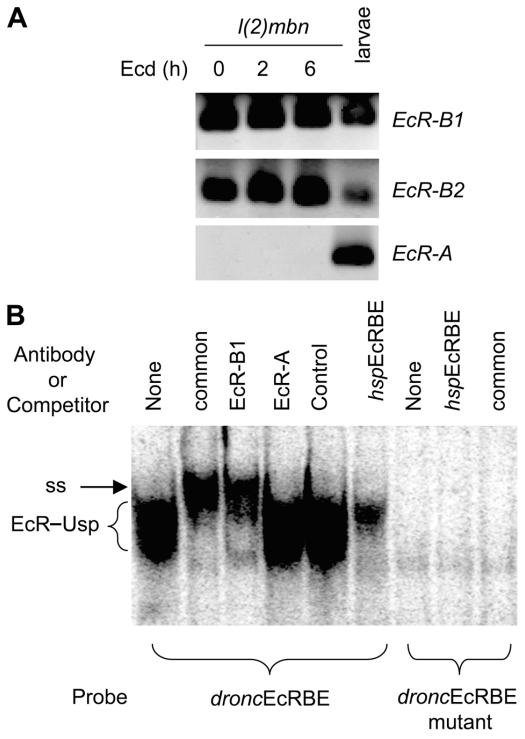

Figure 5. ***l(2)mbn* cells express the EcR-B1 isoform, which binds to the *dronc* promoter.** (A) 10^7 *l(2)mbn* cells were treated with 10 μM ecdysone for the indicated time. RNA was extracted from cells and analyzed by RT-PCR to detect specific EcR isoforms. The last lane of the gel shows EcR isoforms expressed in late third instar larvae/prepupae (120 h after egg laying). (B) 9 μg of nuclear extracts prepared from *l(2)mbn* cells treated with ecdysone for 6 h was incubated with the *dronc*EcRBE or the EcRBE mutant probe for 20 min in the presence of 2 μl of EcR common, EcR-B1, or EcR-A antibody. A mouse control antibody was also used. Complexes were resolved on an acrylamide/TBE gel, dried on 3 MM Whatmann paper, and exposed to Kodak film overnight. EcR–Usp complex (EcR) and supershifted EcR–Usp complex (ss) are indicated.

forms were highly abundant in *l(2)mbn* cells, whereas EcR-A was not detectable (Fig. 5 A). In cDNA prepared from total larvae, EcR-A was clearly evident. To determine which isoform is recruited to the *dronc* promoter, EMSA analysis was performed using isoform-specific EcR antibodies. Nuclear extracts from *l(2)mbn* cells revealed a single complex binding to the *dronc*EcRBE probe, which was entirely supershifted by an EcR antibody that recognizes all EcR isoforms (Fig. 5 B). The EcR-B1 isoform antibody also supershifted the complex, whereas the EcR-A isoform-specific antibody had no effect. As expected, the *hsp*EcRBE oligonucleotide competed most of the complex. Mutation of the *dronc*EcRBE completely inhibited binding of the EcR–Usp complex, and competitor oligonucleotide or EcR common antibody had no effect as no complex bound to this probe (Fig. 5 B). These results clearly demonstrate that *l(2)mbn* cells express EcR-B1, and this isoform is specifically recruited to the *dronc* promoter. As specific EcR-B2 antibodies are unavailable, we were unable to test if this isoform also binds to the *dronc*EcRBE. However, as EcR-B1 specific antibody supershifts most of the complex, it is apparent that most of the binding in the *l(2)mbn* nuclear extracts is due to the EcR-B1 isoform.

EcR-B1 is predominantly expressed in salivary glands and midgut and binds to the *dronc* promoter in these tissues

Because *dronc* is predominantly expressed in midgut and salivary glands from early (2 h after puparium formation in the midgut) and late prepupae (12 h after puparium formation in salivary glands), these tissues were analyzed for EcR expression. RT-PCR analysis showed the expression of EcR-B1 but not EcR-B2 or EcR-A in both salivary glands and midgut (Fig. 6 A). Increasing the number of PCR cycles did also reveal the presence of EcR-B2 (unpublished data). Nuclear extracts prepared from staged animals revealed binding of the EcR–Usp to the *dronc*EcRBE probe from early to late prepupae, which correlates with the stages of midgut and salivary gland cell death (Fig. 6 B). Binding of the EcR complex was abolished upon mutation of the *dronc*EcRBE (Fig. 6 B). Antibody supershift experiments using nuclear extracts from total larvae at the mid prepupal stage showed the binding of EcR-B1 isoform to the EcRBE (Fig. 6 C). Interestingly, EcR-A isoform was also capable of binding to the EcRBE, but given its lack of expression in salivary gland and midgut, this is likely due to the contribution of other larval tissues in the extracts prepared from whole prepupae (Fig. 6 C). When analyzing nuclear extracts from specific tissues, it was evident that EcR-B1 in prepupal salivary gland (12 h) and midgut (2 h) binds to the *dronc* promoter, however no supershift was seen with the EcR-A antibody (Fig. 6 D). Cold EcRBE (*dronc*EcRBE) competitor eliminated binding of the EcR–Usp complex as expected. These results clearly demonstrate that EcR-B1 predominantly binds the *dronc* promoter at least in these two tissues.

*dronc*EcRBE is important for ecdysone-mediated *dronc* transcription

Having established the binding of EcR–Usp to the *dronc* promoter, we determined the significance of direct EcR–Usp binding in ecdysone-mediated *dronc* transcription of this caspase. To assess this, the *dronc* promoter-luciferase reporter constructs with or without EcRBE mutations were introduced into *l(2)mbn* cells, and ecdysone-mediated reporter expression was analyzed. The wild-type promoter was up-regulated in response to ecdysone treatment, and cotransfection of EcR-B1 isoform enhanced ecdysone-mediated transcription (Fig. 7 A). However, mutation of the EcR binding site in the promoter abolished reporter expression in response to ecdysone treatment, and cotransfection of increasing amounts of EcR-B1 had no enhancing effect (Fig. 7 A). Because the region between 2.8–1.1 kb of the promoter is sensitive to TSA treatment (Fig. 1 B), we wished to determine if the EcR mutation inhibits the effects of TSA to determine if the response to TSA is due to the EcRBE or another region of the upstream promoter. The wild-type *dronc* promoter-reporter was activated by TSA treatment, whereas the EcR mutation significantly inhibited this activation (Fig. 7 B). These results demonstrate that the EcR binding site possibly recruits a histone deacetylase for repression of transcription in the absence of ecdysone and is important for ecdysone-mediated *dronc* transcription.

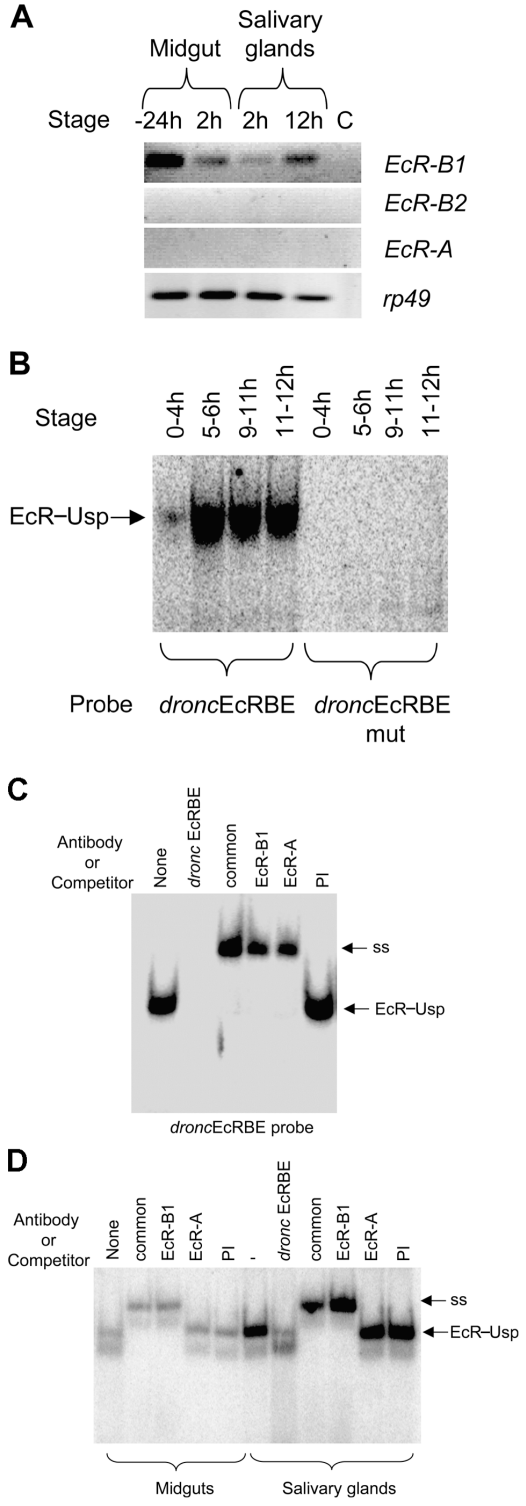

Figure 6. **Salivary glands and midgut express EcR-B1, which binds to *dronc* promoter.** (A) Salivary glands and midgut were dissected from animals at −24, 2, or 12 h relative to puparium formation. RNA was analyzed by RT-PCR to detect EcR isoform expression. *Rp49* was used as a control. (B) 9 μg of nuclear extracts prepared from various staged animals were incubated with the *dronc*EcRBE or the EcRBE mutant probe for 20 min. Complexes were analyzed as in Figs. 4 and 5. EcR–Usp complex and supershift (ss) are indicated. Developmental stages are shown as hours relative to puparium formation. These stages represent early (0–4 h), mid (5–6 h), and late (9–11 h) prepupae and early (11–12 h) pupae. (C) EMSA was performed as in B in the presence of *dronc*EcRBE cold competitor, 2 μl of EcR common antibody, EcR-B1, or EcR-A. EcR–Usp (EcR) and supershifted EcR–Usp (ss) complexes are indicated. (D) EMSA was performed as in C with 6 μg on nuclear extract from salivary glands or midguts from 12 h (salivary gland) and 2 h (midgut) staged prepupae. EcR–Usp and supershifted (ss) complexes are shown. 40 ng of cold competitor (*dronc*EcRBE) was also added where indicated.

*dronc*EcRBE is important for *dronc* expression in specific tissues

We have recently reported that the 2.8-kb *dronc* promoter region contains most necessary elements required for correct spatial and temporal expression (Daish et al., 2003). Interestingly, we also found that the deletion of the 2.8-kb promoter down to 1.1 kb abolished expression in larval/prepupal salivary glands and brain lobes, but not in midgut, suggesting that an element located between 2.8 and 1.1 kb promoter region is necessary for tissue-specific regulation of *dronc* (Daish et al., 2003). One possibility is that the EcRBE described in this work is responsible for this regulation of *dronc*. To test this possibility, we created additional promoter deletions and generated transgenic flies carrying the *dronc* promoter driving expression of the *LacZ* gene. A transgenic construct containing the 1.64-kb *dronc* promoter and including the *dronc*EcRBE was able to drive reporter *LacZ* gene expression in salivary glands, midgut (Fig. 8 A), and brain lobes (not depicted) as assessed by the β-galactoside activity staining of dissected tissues. It should be noted that some premature β-galactoside activity was seen in salivary glands of the 1.64-kb *dronc* promoter-*LacZ* transgenic flies (unpublished data). This finding could imply that the 1.64-kb promoter lacks some control elements that govern precise timing of expression. A 1.33-kb promoter construct that deletes the region just past the EcRBE was unable to drive *LacZ* gene expression in salivary glands (Fig. 8 A) and brain lobes (not depicted). As expected, 1.33 kb of the promoter could efficiently drive reporter expression in the midgut (Fig. 8 A). These data suggested that the region between 1.64 and 1.33 kb, which harbors the EcRBE, is required for *dronc* expression in specific tissues.

The role of direct EcR–Usp–mediated *dronc* regulation was analyzed further in transgenic flies carrying mutations in the *dronc*EcRBE (Fig. 4 A) that abrogate EcR–Usp binding. Transgenic flies carrying mutant EcRBE (in the 2.8 kb *dronc* promoter *LacZ* transgene) were carefully staged together with transgenic flies carrying the wild-type 2.8-kb *dronc* promoter-reporter, and Northern blot analysis was performed comparing *LacZ* and endogenous *dronc* expression. In lines where *LacZ* expression was driven by the wild-type *dronc* promoter, *LacZ* and endogenous *dronc* show low to undetectable levels of expression in midgut and salivary gland at −12 and 10 h (relative to puparium formation), respectively. Both *LacZ* and endogenous *dronc* transcription was up-regulated in the midgut and salivary gland at 2 and 12 h, respectively (Fig. 8 B), at a time when PCD occurs in these tissues. Mutation of the EcRBE did not significantly affect *LacZ* expression in midgut, whereas expression in salivary gland was compromised at 12 h (Fig. 8 B) but detected at later stages (not depicted). As expected, endogenous *dronc* was transcriptionally up-regulated in both tissues (Fig. 8 B).

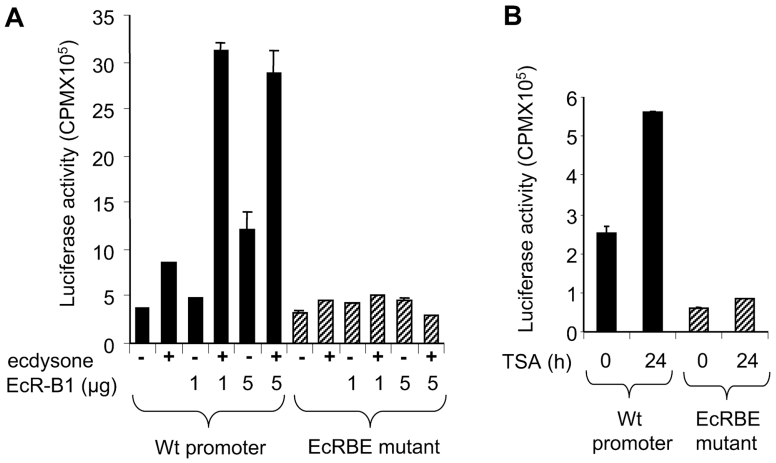

Figure 7. **Mutation of *dronc*EcRBE reduces transcription.** (A) 2.5×10^6 *l(2)mbn* cells were transfected in triplicate with 2 μg of *dronc* luciferase reporter, under the control of the 2.8-kb *dronc* promoter (Wt) or the promoter with the EcRBE mutated (EcRBE mutant). Where indicated, cells were also cotransfected with 1 or 5 μg of the EcR-B1 expression vector. After 24 h, cells were treated with 10 μM ecdysone for 24 h where indicated (+). Cell extracts were prepared and 100 μg of protein assayed in triplicate for luciferase activity. Luciferase activity was subtracted from values obtained from empty luciferase vector transfections alone. (B) Transfections were performed as in A except trichostatin A (TSA) was added at 1 μM as indicated. Error bars represent SD.

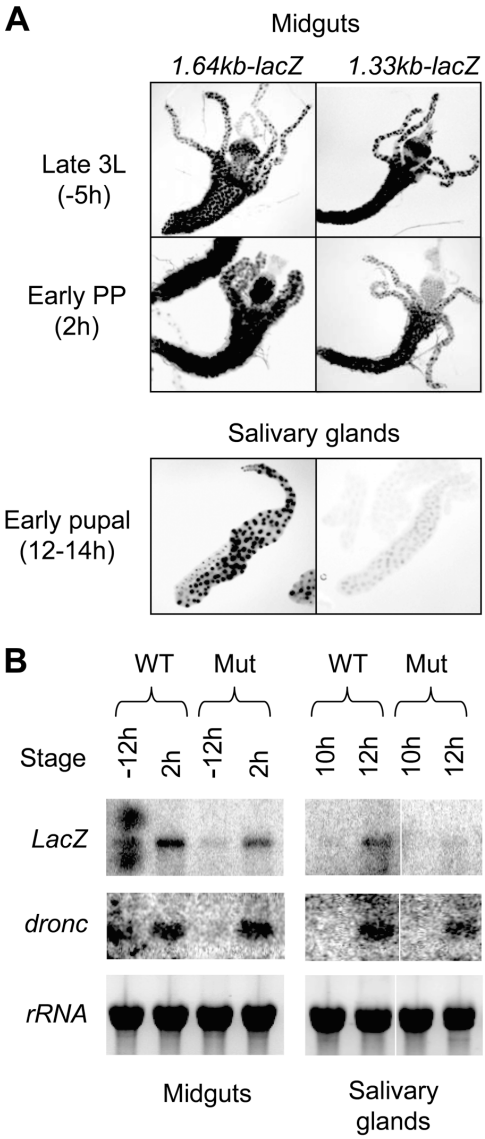

Figure 8. **A region of the *dronc* promoter is required for spatial regulation of expression.** β-Galactoside staining of midguts and salivary glands from *dronc* promoter-reporter transgenic *Drosophila* is shown. Tissues were dissected out at indicated developmental stages and stained with X-gal. (A, top) Qualitatively similar staining patterns for 1.64 kb and 1.33 kb transgenes with expression throughout midgut and gastric cecae from late third instar larvae (3L) and early prepupae (PP). (bottom) Tissue-specific expression between 1.64 and 1.33 kb transgenes in salivary glands from early pupae demonstrating a requirement for the EcRBE for salivary gland-specific expression. Images are representative of multiple lines analyzed. Slight adjustments of brightness and contrast were applied to all images to enhance quality of the presentation. However, this did not obscure or eliminate any information. These images were originally in color. (B) RNA was collected from staged animals carrying the wild-type *dronc* 2.8 kb promoter-*LacZ* transgene (WT) or EcRBE mutant 2.8 kb *dronc* promoter-*LacZ* transgene (Mut) at various times (in hours) relative to puparium formation from midguts (−12 and 2 h) and salivary glands (10 and 12 h) and subjected to Northern blot analysis. Filters were probed with *LacZ* and *dronc*. Slight adjustments of brightness and contrast were applied to images to enhance quality of the presentation. (bottom) A picture of the gel before blotting to demonstrate that approximately equal amounts of intact RNA were present in all lanes. White lines indicate that intervening lanes have been spliced out.

These results demonstrate that the *dronc*EcRBE is required for proper timing of *dronc* expression in the salivary gland.

Discussion

One of the major questions associated with the biological actions of nuclear hormones is how a single hormone can control differentiation, PCD, and proliferation in different tissues. Although useful information has been obtained from mammalian systems, problems of redundancy, different nuclear receptor isoforms, and developmental defects has hampered the ability to decipher the precise mechanisms of nuclear receptor actions. *Drosophila* provides an excellent system to address some fundamental questions associated with nuclear hormone actions. The steroid hormone ecdysone specifically mediates the removal of larval tissues, such as salivary glands and midgut at specific times during development that are then replaced by adult tissues from differentiating progenitor cells (Jiang et al., 1997; Baehrecke, 2000, 2002). We have previously discovered that ecdysone transcriptionally up-regulates *dronc* expression in salivary glands and midgut before PCD in these tissues (Dorstyn et al., 1999a; Daish et al., 2003). The understanding of ecdysone-mediated spatial and temporal regulation of *dronc* expression will greatly assist in deciphering

how nuclear hormones control PCD of specific tissues at precise stages of development.

With the use of luciferase reporter constructs and an ecdysone responsive cell line in this paper, we have established that the promoter region spanning 2.8 to 1.1 kb contains important elements for ecdysone-mediated transcription. We have further shown that this region harbors a putative repressor that acts by recruiting a histone deacetylase as TSA treatment alleviates the repression of transcription. However, TSA has no effect on the 1.1-kb promoter, implying a histone deacetylase is recruited specifically to the 2.8–1.1 kb region. It was further shown that an EcR binding site was present at 1.36 kb from the transcription start site that specifically binds the EcR-B1 isoform in *l(2)mbn* cells and *Drosophila* prepupal salivary gland/midgut nuclear extracts. Functional experiments in *l(2)mbn* cells have established that this site is vital for *dronc* transcription as mutation reduces ecdysone-mediated activation and transactivation by EcR-B1. Due to the anomalies associated with cell lines, the importance of the EcRBE was also assessed in transgenic flies. The results are supported by our recent findings that the 2.8-kb promoter contains all necessary elements for correct spatial regulation in *Drosophila* and deleting the promoter to 1.1 kb (eliminating the EcR binding site) renders it inactive in salivary glands and brain lobes (Daish et al., 2003). This finding was further supported by two in vivo approaches. First, the importance of *dronc*-EcRBE was demonstrated by deletion just before the EcR binding site, which had no abrogating effect on expression, whereas deletion of the EcR binding site eliminated transcription in both salivary glands and brain lobe without any effect in the midgut. In addition, specific mutation of the EcRBE delayed expression in salivary glands without affecting midgut expression.

The lack of effect of EcRBE mutation in the midgut can be explained in many ways. For example, a different chromatin structure along the *dronc* promoter in this tissue may allow other factors to play a dominant role for *dronc* expression. The chromatin structure surrounding the EcR binding site in the midgut may also preclude EcR–Usp from binding to EcRBE (chromatin effects are not taken into consideration in EMSA experiments). Coactivators play a key role in modifying chromatin in nuclear hormone-mediated transcription. For example, CARMER, a *Drosophila* histone methyl transferase, required for ecdysone-induced expression of cell death genes in *l(2)mbn* cells (Cakouros et al., 2004), may have some role in tissue-specific gene expression. Alternatively, a midgut-specific transcription factor may inhibit binding of EcR–Usp to the *dronc* promoter. Our results show that the *dronc*EcRBE has a lower affinity for the EcR–Usp than the consensus site (Fig. 4 B), and expression analysis shows a decrease in EcR-B1 expression at the time of *dronc* expression in the midgut but an increase in expression in the salivary gland (Fig. 6 A). The lower affinity and lower EcR expression suggests that insufficient amounts of EcR bind to the promoter in the midgut, whereas the increase in EcR-B1 expression in salivary glands overcome this problem. In fact, this result is observed in EMSA performed with extracts from tissues (Fig. 6 D) as identical levels of nuclear extracts show better binding from salivary gland extracts when compared with midgut. In any case, our results show that the EcRBE is important for expression of *dronc* specifically in salivary gland and brain lobes, but not in midgut (Fig. 9).

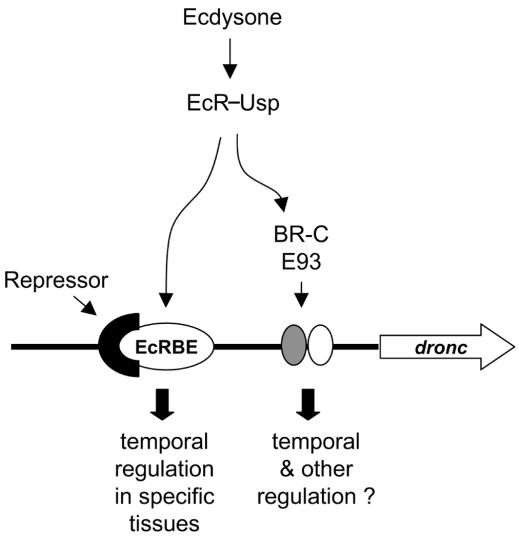

Figure 9. **A model of ecdysone-mediated regulation of *dronc* expression.** Ecdysone binds its heterodimeric receptor EcR–Usp and activates *dronc* expression in specific tissues by directly interacting with an EcRBE in the promoter. Recruitment of a potential repressor to EcRBE region is likely to be required for the correct temporal regulation of *dronc* expression. EcR–Usp also regulates *dronc* transcription via ecdysone-induced transcription factors BR-C and E93, which may regulate temporal expression. Because E93 is required for expression of *dronc* in both larval salivary gland and midgut (Lee et al., 2000), it may also play a role in the spatial regulation of *dronc* expression.

Although the EcRBE is important for proper salivary gland *dronc* transcription, it is possible that it does not function alone but in cooperation with other factors that might govern tissue-specific expression. Numerous examples of this already exist such as the dGATAb transcription factor which binds to three binding sites flanking a EcR–Usp binding site of the Fbp1 promoter directing fat body-specific transcription (Brodu et al., 1999). Studies on Sgs4 gene transcription, which is specifically expressed in larval salivary glands, have revealed that its tissue-specific expression is governed by the Forkhead transcription factor in this tissue (Lehmann and Korge, 1996). In light of this, we have previously shown that BR-C binding sites exist in the *dronc* promoter (Cakouros et al., 2002), and given their similarity to Forkhead binding sites, it is possible that in salivary glands these sites also bind Forkhead. However, this possibility remains to be tested.

Our data show that both EcR-B1 and EcR-A isoforms are able to bind the *dronc* promoter EcRBE. Because EcR-A isoform is not expressed in *l(2)mbn* cells or larval/pupal midgut and salivary glands (or expressed at levels below detection), it is unlikely to play an important role in ecdysone-mediated *dronc* transcription and PCD. Consistent with this, previous works have shown that ectopic expression of the EcR-A isoform in EcR-B1–deficient salivary glands does not restore their ability to respond to ecdysone (Bender et al., 1997).

In mammals it is well documented that nuclear receptors recruit corepressors in the absence of ligand and specific co-

activators in the presence of ligands (Shibata et al., 1997). These coactivators can be tissue and promoter specific, and bind to selected receptor isoforms. In this work, we have provided evidence of the possible recruitment of a repressor to the upstream *dronc* promoter region. This repressor seems to recruit a histone deacetylase as assessed by sensitivity to TSA. However, TSA sensitivity is reduced when the EcRBE is mutated, indicating that the EcR–Usp binding is partly responsible for recruiting this repressor. If a repressor was recruited by EcR–Usp, then the mutation of EcRBE site should show increased basal expression. However, this increase was not observed. One possible reason for this lack of increased activity is because increased promoter activity is likely to be seen when the corepressor is inactivated or eliminated, enabling coactivators to bind to the EcR–Usp, increasing basal activity. However, in the absence of EcR binding to the promoter, coactivators are unable to be recruited to the promoter, and there will therefore be no increase in basal activity. Given that these results are suggestive of the actions of a corepressor, more detailed experiments are being undertaken to identify the recruitment of such a repressor.

Overall, we have demonstrated that the EcR-B1 isoform is directly recruited to the *dronc* promoter and is required for proper temporal *dronc* transcription in specific tissues. The data presented here forms the foundation of future work to address important questions associated with spatio-temporal gene expression and PCD and the role of nuclear hormones in these processes. We believe that the *dronc* promoter provides an important tool for such studies.

Materials and methods

Cell culture

l(2)mbn cells (a gift from A. Dorn, Johannes Gutenberg University, Mainz, Germany; Ress et al., 2000) were grown in Schneider's medium supplemented with 10% FBS. 2.5×10^6 per well cells were seeded in 6-well plates in triplicate. Where necessary, 10 μM ecdysone (Sigma-Aldrich) was added for the desired time. Cycloheximide was used at 10 μg/ml. TSA (Sigma-Aldrich) was used at 1 μM. Cell viability was assessed by trypan blue exclusion.

Northern blotting and RT-PCR

Total RNA was extracted using Trizol reagent (Life Technologies), and 15–20 μg was analyzed by Northern blotting using ^{32}P-labeled probes as described previously (Colussi et al., 2000; Cakouros et al., 2002). For RT-PCR, 2 μg RNA was used to generate cDNA using Superscript II reverse transcriptase (Invitrogen). cDNA was diluted 1:10 and 1 μl was used in a standard PCR reaction. PCR conditions have been described previously (Daish et al., 2003).

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 DRONC antibody (Quinn et al., 2000; Dorstyn et al., 2002) was used at a 1:300 dilution. Purified antibodies to EcR common, EcR-B1, and EcR-A from the hybridoma bank were used at a 1:2,000 dilution. Secondary HRP-conjugated anti–mouse antibody (Amersham Biosciences) was used at a 1:2,000 dilution. Signals were detected using the ECL system (Amersham Biosciences).

Expression constructs

The luciferase reporter pxpGDR2.8kbLuc (generated by P. Colussi, Hanson Institute, Adelaide, Australia) contains a 2.8-kb region of the *dronc* promoter upstream of the transcription start site up to the ATG cloned into the luciferase reporter vector pxpG (provided by P. Cockerill, Hanson Institute, Adelaide, Australia). Deletions of the promoter were made by PCR amplification from 1.1 kb and 0.54 kb relative to transcription start site up to the ATG site and cloned into the pxpG luciferase reporter vector. EcR-B1 expression construct was provided by M. Bender (University of Georgia, Athens, GA).

Preparation of nuclear extracts and EMSA

7.5×10^6 *l(2)mbn* cells were pelleted, washed once in PBS, resuspended in 800 μl of buffer A (10 mM Hepes, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, and Complete™ protease inhibitors from Roche), and placed on ice for 15 min. 0.1% NP-40 was added, and cell suspension was vortexed for 30 s and centrifuged at 13 K for 30 s at 4°C. Nuclear pellets were resuspended in 80 μl of buffer C (10 mM Hepes, pH 7.6, 400 mM NaCl, 7.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and Complete™) and incubated on ice for 40 min while shaking. Extracts were centrifuged for 5 min at 13 K, and supernatant were aliquoted and frozen at −70°C. Nuclear extracts were prepared from staged larvae ranging from 120–132 h AEL by homogenizing 50 larvae in 300 μl of buffer A and removed to a fresh tube in a total of 600 μl devoid of larval debri. After incubation on ice for 15 min, 10 μl of 10% NP-40 was added, lysates were vortexed for 30 s, and spun at 13 K for 30 s. Nuclear pellets were resuspended in 60–100 μl of buffer C and incubated on ice with shaking for 1 h. Lysates were centrifuged for 5 min at 13 K at 4°C and supernatant frozen as aforementioned. EMSA was performed by incubating 7–12 μg (4 μl) of nuclear extracts in binding buffer (20 mM Hepes, pH 7.9, 80 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 10% glycerol) containing 1mg/ml BSA and 1 μg of poly dI/dC for 5 min on ice. Where needed, 2 μl of murine EcR-B1, EcR common, EcR-A antibody, or a control antibody was also added to the nuclear extracts and incubated on ice before the addition of labeled probe. Where indicated, 40 ng of cold competitor oligonucleotide was also included. 0.2 ng of labeled probe was added and incubated on ice for a further 20 min. 5 μl of 5× loading buffer was added, and samples were electrophoresed on a 5% polyacrylamide/0.5×TBE gel. The gel was dried down onto 3 MM Whatmann paper and exposed to Kodak X-ray film.

Transfection and luciferase assay

2 μg of pxpGDR2.8kbLuc, pxpGDR2.8kbEcRmutLuc, pxpGDR1.1kbLuc, or pxpGDR0.54kbLuc was transfected using Cellfectin alone or with 1–5 μg of EcR-B1 expression constructs. Equal amounts of DNA were used with the pIE1-4 expression vector. DNA in a total volume of 100 μl in Schneider media (without FBS) was added to Cellfectin (2:9 ratio) in 100 μl of total media devoid of FBS and incubated at RT for 15 min. 800 μl of serum-free medium was added and 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 were allowed to recover for 24 h. Where needed, 10 μM ecdysone was added for 24 h. Cells were harvested 48 h after transfection, resuspended in lysis buffer (100 mM phosphate buffer, pH 7.8, 10 μM EDTA, and 2 mM DTT), and frozen three times in liquid nitrogen. After centrifugation for 5 min at 13 K, the supernatant was analyzed for luciferase activity. 60–100 μg of protein was assayed in 200 μl of assay buffer (100 mM phosphate buffer, 8 mM $MgSO_4$, 2 mM DTT, 0.75 mM ATP, and 0.175 mM coenzyme A) using an illuminometer (Packard Instrument Co.).

Generation and analysis of *dronc* reporter lacZ lines

1.64 kb and 1.33 kb of the *dronc* promoter were PCR amplified from *Drosophila* genomic DNA using Expand High Fidelity PCR System (Roche) with the primer sets DrPrF3cBglII (5′ CCG AGA TCT ATG TAC GTT ATG TTA TAG TAA GTG TA 3′); DrPrR1BglII (5′ CGG AGA TCT CCG GAT ATG GCT TCC ACG CGT 3′) and DrPrF3eBglII (5′ CGA AGA TCT AAT TGT GTA CAA CTA AAG GAA 3′); DrPrR1BglII, respectively. PCR products were cloned into pGem-T easy (Promega), and then subcloned into the BglII site of the p-element transformation lacZ reporter vector pCaSpeR-NLSlacZ (provided by Carl Thummel, Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT) after BglII digestion. The 2.8-kb *dronc*-LacZ reporter construct has been described previously (Daish et al., 2003). The EcRBE mutations were introduced by PCR using standard techniques. Clones were sequenced for correct orientation, and transgenic flies were generated and transgenes mapped by established techniques. Animals were staged and tissues stained for β-galactosidase activity as described previously (Daish et al., 2003). In brief, third instar larval stages were determined by the gut clearance technique after growth of animals on bromophenol blue–supplemented food. Prepupal and pupal stages were attained by collecting newly pupariated animals from clear gutted third instar larvae populations every 30 min and ageing at 25°C to desired stages before collection and analysis. Images were acquired using a micro-

scope (model SZ40; Olympus) set at 3–4× objective with a 2× adaptor lens (110AL2x), fitted with a digital camera (model DP11; Olympus). Images were cropped and processed using Adobe Photoshop software.

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