Ecdysone receptor directly binds the promoter of the \textit{Drosophila} caspase \textit{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 \textit{Drosophila} initiator caspase, is transcriptionally regulated by ecdysone during development. We demonstrate here that the \textit{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 \textit{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 \textit{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 \textit{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 \textit{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).
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 BFTZ-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 expression (Jiang et al., 2000, Cakouros et al., 2002). BR-C and E74A are also required for the optimal induction of these factors

**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](https://www.jcb.org)

Figure 1. **Upstream dronc promoter is responsive to ecdysone and TSA.** (A) 2.5 × 10⁵ l(2)mbn cells were transfected in triplicate with 2 μg of dronc luciferase reporter, pspDR2.8kbLuc, pspDR1.1kbLuc, or pspDR0.54kbLuc. 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. 10⁵ 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 assessed 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 corepressors 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.

dronc expression is regulated by EcR–Usp

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 hspEcRBE 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 hspEcRBE could be competed out with the hspEcRBE. Increasing amounts of the droncEcRBE oligonucleotide also competed the EcR–Usp complex, however higher amounts were needed to abolish binding of EcR–Usp.
to hspEcRBE. The mutant oligonucleotide failed to compete for the EcRBE. (Fig. 4 B). The ability of the droncEcRBE 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 competes with the hspEcRBE oligonucleotide. Mutation of the droncEcRBE rendered it incapable of binding EcR–Usp (Fig. 4 C).

**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-
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 droncEcRBE 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 droncEcRBE (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 prepupa (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 (droncEcRBE) 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.

**droncEcRBE 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.
droncEcRBE 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 X-galactoside activity staining of dissected tissues. It should be noted that some premature X-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 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 droncEcRBE (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-LacZ 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 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 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 droncEcRBE 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 droncEcRBE cold competitor,
**Figure 7.** Mutation of **dronc**EcRBE reduces transcription. (A) $2.5 \times 10^5$ [L2]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 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.
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 lobes 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).

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

1/2mbn cells (a gift from A. Dorn, Johannes Gutenberg University, Mainz, Germany; Ross et al., 2000) were grown in Schneider’s medium supplemented with 10% FBS. 2.5 \times 10^7 per well cells were seeded in 6-well plates in triplicate. Where necessary, 10 μM ec dysone (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 32P-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 1:3000 dilution. Purified antibodies to EcR coregulator and EcR-A (4C11, H9262, G11003) from the hybridoma bank were used at a 1:2000 dilution. Secondary HRP-conjugated anti–mouse antibody (Amersham Pharmacia Biotech) was used at a 1:3000 dilution. Affinity-purified DRONC antibody (Quinn et al., 2000; Dorstyn et al., 2000) was used at a 1:300 dilution. Primary DRONC antibody was generated in rabbits against synthetic peptides corresponding to the upstream 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.

Transfection and luciferase assay

2 μg of pxpGDr2.8kbLuc, pxpGDr2.8kbEcRmutLuc, pxpGDr1.1kbLuc, or pxpGDr0.5kbLuc was transfected using Cellfectin alone or with 1–5 μg of Ec-R1 expression constructs. Equal amounts of DNA were used with the pE14 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. 500 μl of serum-free medium was added and overlaid onto 2.5 × 10⁶ cells in 6-well plates. Cells were incubated with 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, 100 μM ec dysone was added for 24 h. Cells were harvested 48 h after transfection

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 DrPrF3 and BglII (5’ CCG AGA TCT ATG TAC GTG TTA TAG TAA GTC TTA 3’); DrPrR1 and BglII (5’ CCG AGA TCT CCG GAT ATG GCT TCC AGG CGT 3’); DrPrR3 and BglII (5’ CGA AGA TCG TGT GTA CAA AAG GAA 3’); DrPrR1 and BglII, 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., 2004). 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 beemodified food. Preparation of prepupal and pupal stages was achieved by collecting newly pupariated animals from clear gutted third instar larval 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 (110A2x), fitted with a digital camera (model DP11; Olympus). Images were cropped and processed using Adobe Photoshop software.

We thank Drs. Carl Thummel, Peter Cockerill, Paul Colussi, Augustus Dorn, and Michael Bender for reagents and cell lines.

This work was supported by the National Health and Medical Research Council of Australia. T. Daish is the holder of a Dawes Postgraduate Scholarship.

Submitted: 12 November 2003
Accepted: 3 May 2004

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