Cell Death and Differentiation (2003) 10, 1348–1356

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Distinct promoter regions regulate spatial and temporal expression of the *Drosophila* caspase *dronc*

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Received 05.6.03; revised 16.7.03; accepted 18.7.03; published online 29 August 2003
Edited by E Baehreckle

Abstract

DRONC is an apical *Drosophila* caspase essential for programmed cell death during fly development. During metamorphosis, *dronc* gene expression is regulated by the steroid hormone ecdysone, which also regulates the levels of a number of other critical cell death proteins. As DRONC protein levels are important in determining caspase activation and initiation of cell death, we have analyzed the regulation of the *dronc* promoter using transgenic flies expressing a *LacZ* reporter gene under the control of the *dronc* promoter. Our results indicate that *dronc* expression is highly dynamic during *Drosophila* development, and is controlled both spatially and temporally. We demonstrate that while a 2.3 kb *dronc* promoter region contains most of the information required for correct gene expression, a 1.1 kb promoter region is expressed in some tissues and not others. We further demonstrate that during larval–pupal metamorphosis, two ecdysone-induced transcription factors, Broad-Complex and E93, are required for correct *dronc* expression. Our data suggest that the *dronc* promoter is regulated in a highly complex manner, and provides an ideal system to explore the temporal and spatial regulation of gene expression driven by nuclear hormone receptors.

Cell Death and Differentiation (2003) **10,** 1348–1356, doi:10.1038/sj.cdd.4401312
Published online 29 August 2003

Keywords: ecdysone; caspase expression; transcription; salivary glands

Abbreviations: PCD, programmed cell death; β-gal, β-galactosidase; CPRG, chlorophenol red β-D-galactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; AEL, after egg laying; L2, second instar larvae; L3, third instar larvae; PP, prepupae

Introduction

In metazoans, programmed cell death (PCD), often by a morphologically distinct process called apoptosis, is essential for removing unwanted cells.[1] The execution phase of PCD requires the activation of a unique class of proteases, termed caspases.[2,3] The initial activation of caspases requires specific adaptor molecules that mediate procaspase oligomerization.[4,5] Once activated, apical caspases process and activate downstream effector caspases which cleave a large number of cellular proteins in condemned cells to bring about characteristic dismantling of the cell architecture.[2,3,6] As key cell death molecules, including caspases, are present in healthy cells, it is generally believed that apoptosis is primarily regulated post-transcriptionally. However, many components of the core apoptosis machinery, including some caspases, are transcriptionally regulated during cell death signaling, suggesting that the levels of the prosurvival and proapoptotic factors in the cell may be crucial to activate the apoptotic program.[1] Consistent with this, various signals such as cytotoxic insults, hormones, and growth factors regulate the activation of the death program by controlling the balance between prosurvival and proapoptotic proteins of the core cell death machinery.[1] To understand cell death regulation, it is thus essential to understand the role of transcriptional control in the execution of apoptosis.

In *Drosophila melanogaster*, pulses of the steroid hormone 20-hydroxyecdysone (ecdysone) throughout fly development regulate molting, cell proliferation, differentiation, and PCD in a highly controlled manner.[1,7,8] Importantly, during metamorphosis, ecdysone is a primary regulator of PCD in larval tissues such as the salivary glands and midgut.[1,9–11] Ecdysone binds to its heterodimeric EcR/Usp receptor, and transcriptionally regulates a number of primary response genes. During the transition of larva into pupa, a late third instar larval (L3) ecdysone pulse signals puparium formation, followed by a second pulse approximately 12 h later which initiates head eversion and pupal development.[1,9–11] Cell death in the larval midgut begins in response to the late larval ecdysone pulse, whereas salivary glands undergo removal around 15 h later in response to the late prepupal hormone pulse.[9] The regulation of PCD by ecdysone is complex and involves EcR/Usp itself and a number of induced transcription factors including β*FTZ-F1*, *BR-C*, *E74*, *E75*, and *E93*.[1,12–19] Several of these factors regulate the expression of cell death/survival genes, including *rpr*, *hid*, *dark*, *dronc*, *diap1*, and *diap2*.[1,12]

In *Drosophila,* the main components of the cell death machinery, including caspases, Bcl-2 homologues, and an Apaf-1 homologue, are conserved.[1,20–34] Among the seven caspases in *Drosophila*, DRONC is the main initiator caspase that appears to be functionally similar to CED-3 in *Caenorhabditis elegans* and caspase-2/caspase-9 in mammals.[23,35–37] DRONC was the first caspase shown to be regulated by a steroid hormone.[23] In the salivary glands and midgut, *dronc* expression is dramatically upregulated by ecdysone prior to their removal during metamorphosis.[23] *dronc* is also rapidly upregulated by ecdysone in *Drosophila l(2)mbn* cells, which undergo apoptosis in response to ecdysone treatment.[38] Furthermore, DRONC is essential for

ecdysone-mediated apoptosis of *l(2)mbn* cells.[38] Although the importance of *dronc* regulation is well established for ecdysone-mediated cell death, the mechanisms of *dronc* regulation remain poorly understood. In the present study, we have analyzed the regulation of the *dronc* promoter, and we report that different regions of the *dronc* promoter are involved in ecdysone-dependent and -independent temporal and spatial regulation of *dronc* expression during fly development.

Results

dronc promoter-driven LacZ expression in embryos and adult ovaries

We have previously shown that the *dronc* transcript is expressed highly in embryos, at very low levels in first instar (L1) to mid-L3 stages, and at higher levels in the midgut and salivary glands from late L3.[23] Low levels of *dronc* expression are also seen throughout the L3 eye discs and in brain lobes, whereas strong expression is evident in adult egg chambers.[23] To study the *in vivo* regulation of *dronc* expression, we generated a number of transgenic constructs containing various lengths of the *dronc* promoter region fused to the LacZ gene containing a nuclear localization signal (Figure 1). This allowed us to monitor the expression of the reporter LacZ, the product of which accumulates in the nuclei of cells. Transgenic *Drosophila* lines containing 0.54, 1.1, 2.3, and 2.8 kb of the *dronc* promoter region were generated. As the expression patterns of 2.3 and 2.8 kb promoter-LacZ were similar, only the results with 2.8 kb lines, which were analyzed in detail, are presented in this paper. Several lines obtained from the same construct were analyzed to ensure that the reporter expression was not affected due to the transgene integration site.

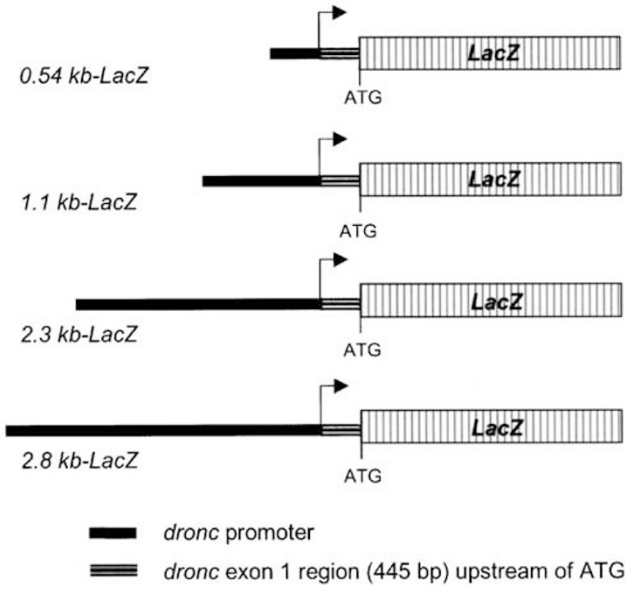


Figure 1 *dronc* Promoter-*LacZ* constructs used to generate transgenic flies. Various lengths of the promoter upstream of the *dronc* transcription start site (shown by an arrow) were cloned upstream of a *LacZ* reporter gene containing a nuclear localization signal, into the pCaSpeR-4 *Drosophila* transformation vector. All constructs also contain the 445 bp noncoding region of the *dronc* exon 1. In all constructs, the initiator ATG codon is the natural *dronc* initiation codon

An analysis of β-galactosidase (β-gal) activity in early embryos collected at 0–4 h after egg laying (AEL) stained with X-gal demonstrated that while the 1.1 and 2.8 kb promoter-LacZ lines showed β-gal expression, the 0.54 kb promoter-LacZ lines did not (Figure 2a). The 2.8 kb construct showed a somewhat higher expression of β-gal in embryos 4–8 h AEL (Figure 2a), suggesting that while 1.1 kb may be driving the basal expression of the reporter, 2.8 kb is required for maximal promoter activity upon commencement of the zygotic gene expression. We have previously shown that the *dronc* transcript is maternally deposited in the early embryos.[23] As zygotic expression of genes in *Drosophila* does not begin until around stage 5 ($\sim$3 h AEL), β-gal expression seen in embryos 0–4 h AEL may largely represent maternally deposited LacZ. Therefore, we tested the activity of β-gal in egg chambers of adult ovaries from transgenic lines. As in embryos, no β-gal expression was evident in lines carrying the 0.54 kb-LacZ construct (Figure 2b). However, both 1.1 and 2.8 kb promoters were able to drive LacZ expression in nurse cells (Figure 2b), suggesting that the 1.1 kb promoter was sufficient for β-gal expression. As nurse cells dump their contents into the developing oocyte, the β-gal activity in 0–4 h embryos with 1.1 and 2.8 kb promoter transgenes may primarily be due to the maternally deposited transcript. As the activity of the 2.8 kb-LacZ transgene is higher in 4–8 h embryos, we conclude that 2.8 kb promoter region is required to direct the limited zygotic expression of *dronc* in embryos.

Regulation of *dronc* promoter in the midgut and salivary glands during metamorphosis

As a major ecdysone-mediated upregulation of *dronc* expression occurs in the larval salivary glands and midgut just prior to their removal, we analyzed the expression of the promoter-LacZ transgenes in these tissues. In midguts dissected from late L3 ($\sim$115 h AEL) or early prepupae ($\sim$122 h AEL), 1.1 and 2.8 kb promoter-LacZ transgenes were expressed efficiently, however 2.8 kb promoter-driven LacZ expression is lower in gastric caecae (Figure 3a), indicative of different spatial regulation of the two promoter constructs due to heterogeneity in this tissue. The 0.54 kb-LacZ promoter was unable to drive the reporter gene expression (Figure 3a). Note that early prepupal midgut is undergoing PCD at this stage (Figure 3a, lower panel). Interestingly, in salivary glands isolated from late L3 or early prepupae, both 0.54 and 1.1 kb promoter-LacZ transgenes failed to express, however, the 2.8 kb promoter-LacZ was expressed efficiently (Figure 3b, upper two panels). As salivary glands undergo histolysis $\sim$15 h later than midgut, we also analyzed the expression of the 1.1 and 2.8 kb-LacZ transgenes in salivary glands isolated from early pupae (132–134 h AEL). As in earlier stages, only 2.8 kb promoter was able to drive the reporter expression in salivary glands from this late stage (Figure 3b, lower panel). A 2.3 kb promoter-LacZ transgene showed a pattern of β-gal expression similar to that of the 2.8 kb promoter-LacZ construct (data not shown). These results suggest that while the 1.1 kb promoter is sufficient to drive the expression of *dronc* in the midgut, at least 2.3 kb of the promoter is required for driving the expression in salivary glands. These results

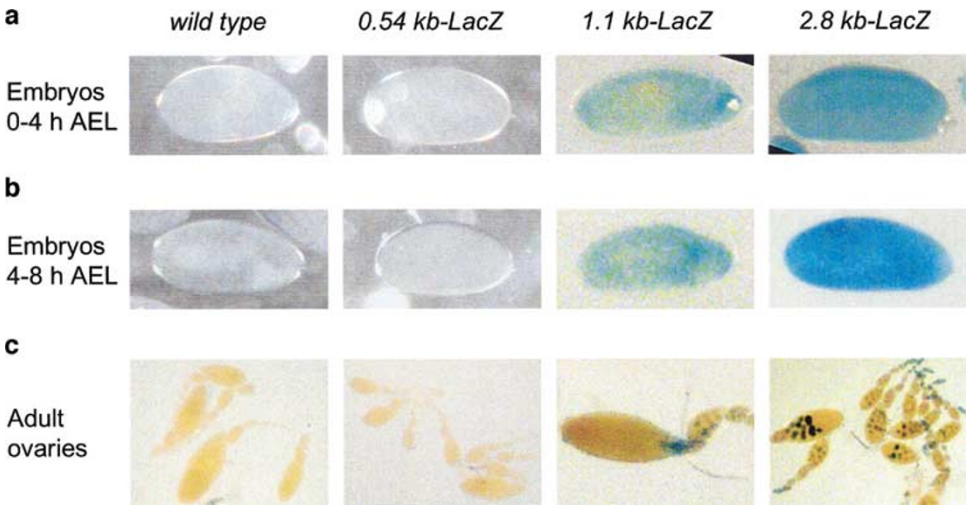


Figure 2 Expression of various *dronc* promoter-*LacZ* transgenes in wild-type (W^{1118}) or transgenic *Drosophila* embryos (**a**, **b**) and egg chambers (**c**). In (**a**, **b**), the time shown on the left represents h AEL. Dechorionated mixed-stage embryos 0–4 h AEL (**a**) or 4–8 h AEL (**b**), or dissected ovaries (**c**) from adult flies were stained with X-gal, as described in Materials and Methods. After mounting, the stained samples were photographed using an Olympus microscope fitted with a digital camera. β-Gal staining in transgenic flies containing the 1.1 and 2.8 kb promoter transgene appears diffused in embryos (**a**, **b**), and as distinct nuclear spots in the nurse cells in the egg chambers (**c**). In the wild-type and 0.54 kb promoter-*LacZ* embryos (**a**, **b**), no X-gal staining was apparent. Phase-contrast microscopy was used to visualize these unstained embryos. All the reporter lines are homozygous for the transgene. Multiple lines derived from each transgenic construct were analyzed for β-gal activity. The figure shows representative examples of transgene expression

also indicate that a region between 1.1 and 2.3 kb of the promoter is essential for *dronc* expression in salivary glands. We noted that in salivary glands, reporter expression driven by the 2.8 kb promoter construct was somewhat higher and earlier in development when compared to endogenous *dronc* expression (data not shown). This observation suggests that additional control elements may be necessary for the precise temporal regulation of *dronc* expression in salivary glands.

Expression of the promoter-LacZ transgenes in larval brain lobes

Our previous studies have shown that endogenous *dronc* is expressed in the L3 brain lobes, salivary glands, and midgut.[23] Given the spatial pattern of *dronc* promoter function in the salivary gland and midgut, we further analyzed β-gal expression in the larval brain lobes of promoter-LacZ transgenic lines. As shown in Figure 4, neither 0.54 or 1.1 kb promoter was able to express the reporter in brain lobes, but the 2.8 kb promoter (also the 2.3 kb promoter – data not shown) could efficiently drive LacZ expression. This suggests that, as in larval/prepupal salivary glands, a 2.3 kb promoter region is required for expression in this tissue.

Temporal regulation of the *dronc* promoter

In an effort to ascertain the minimal promoter requirement for correct temporal regulation throughout *Drosophila* development, we compared expression of the LacZ transgene with that of endogenous *dronc*. An analysis of *dronc* and LacZ transcript in whole animals during development suggested that the pattern of the 2.8 kb promoter-driven LacZ expression is similar to that of endogenous *dronc* (Figure 5a). This suggests that the 2.8 kb of *dronc* promoter contains most information required for the temporal regulation of *dronc* expression.

We further analyzed the 1.1 and 2.8 kb promoter activity in larval and prepupal stages, by assaying for β-gal activity in whole animals at various stages of development (Figure 5b). Consistent with low *dronc* expression, the 2.8 kb promoter-LacZ-driven β-gal expression was low in the second instar larvae (L2) which increased significantly at 96 h AEL, followed by a sharp decrease and then steady increase from 120 h AEL, presumably in response to the late larval pulse of ecdysone which punctuates the commencement of metamorphosis. Interestingly, 1.1 kb-LacZ-driven expression of β-gal was somewhat higher in the L2, peaked early at 90 h AEL, and then declined thereafter until the prepupal ecdysone pulse (Figure 5b). The elevated activity of the 1.1 kb promoter at 90 h AEL suggests that this region of the promoter lacks a control mechanism normally required to suppress *dronc* expression at this stage of development. In other words, our data suggest that the region of *dronc* promoter between 1.1 and 2.8 kb is regulated by a temporally controlled transcriptional repressor. This is supported by the observation that the 2.8 kb promoter is less responsive to the mid-L3 ecdysone pulse which initiates the larval wandering stage.[7]

Role of E74A, BR-C, and E93 in the regulation of *dronc* expression

To analyze the roles of ecdysone-induced genes in regulating *dronc* expression, we expressed the *dronc* promoter-LacZ transgenes in a background null for E74A, BR-C, and E93, three EcR/Usp-regulated factors implicated in the regulation of PCD during larval/pupal metamorphosis.[11,16–19] As the midgut is removed about 15 h earlier than salivary glands in response to the late larval ecdysone pulse that initiates pupariation,[10,12] it was dissected out from late L3 ($\sim$114 h

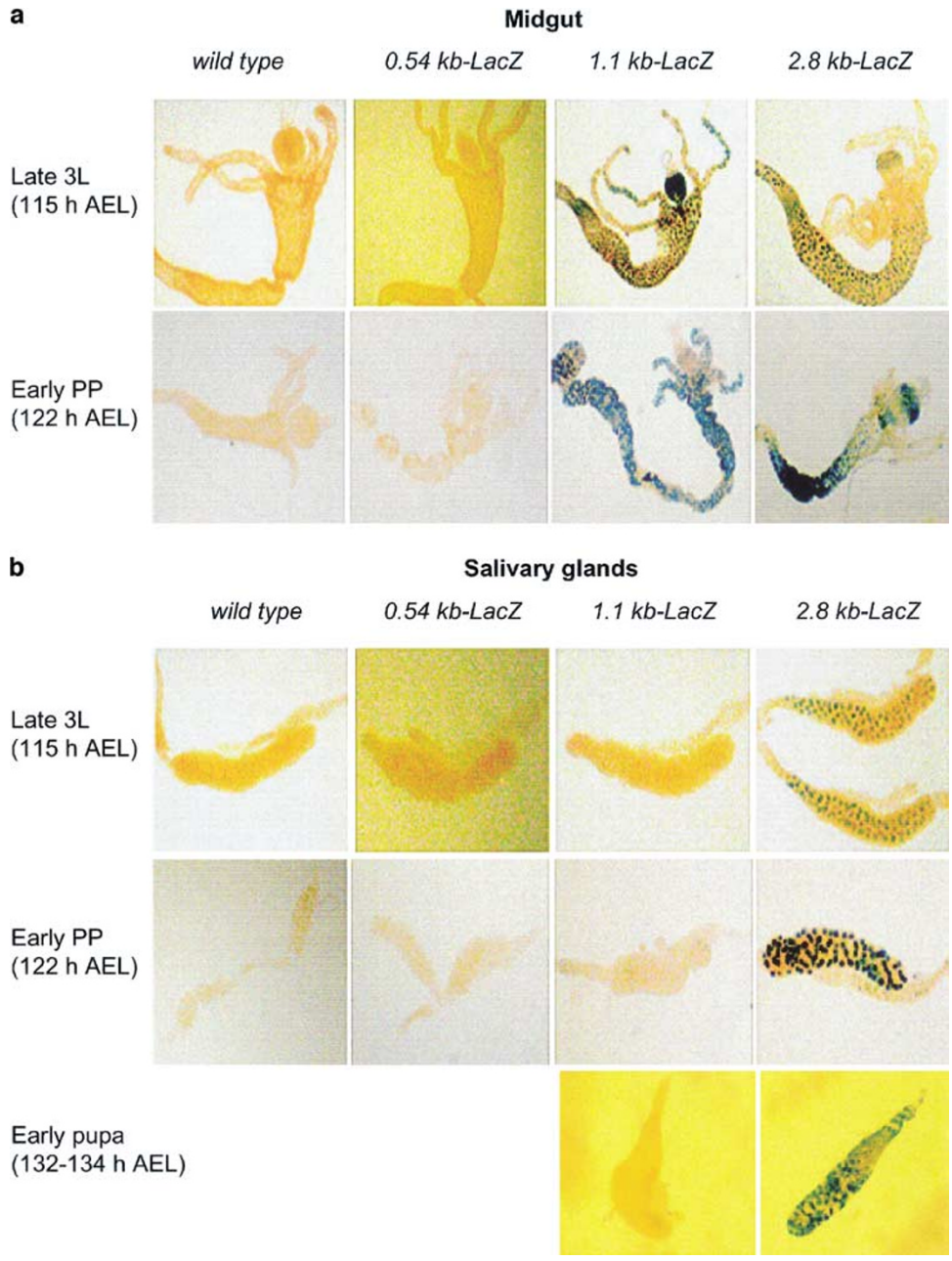


Figure 3 Expression of various *dronc* promoter-*LacZ* transgenes in the midgut (**a**) and salivary glands (**b**) from the wild-type and transgenic animals. The midgut and salivary glands were dissected out from late L3 (~115 h AEL) and early prepupae (~122 h AEL), and stained with X-gal. Salivary glands, which undergo histolysis later than midgut, were also dissected out from an early prepupal stage (132–134 h AEL) and stained with X-gal (bottom panel in (**b**)). Note that the prepupal midguts are undergoing PCD, whereas salivary glands, which are deleted around 15 h later, are still fully intact at this stage. In the midgut, the prepupal 2.8 kb promoter-*LacZ* reporter expression pattern is similar to that observed in late L3. No β-gal staining was observed in the midgut and salivary glands from wild-type controls or in 0.54 kb promoter-*LacZ* transgenic lines

AEL) and early (white) prepupae (~120–125 h AEL). Salivary glands, which undergo histolysis in response to the prepupal ecdysone pulse,[10,12] were dissected out from late L3, early prepupae, and early pupae (~132 h AEL).

We used two BR-C mutants, npr1[41] and rbp5.[11] npr1 mutants are null for all BR-C isoforms, and fail to initiate pupariation.[41] The rbp5 mutant is deficient in the BR-C Z1 isoform, shows a defect in the removal of the larval salivary gland, and fails to develop beyond early pupae.[11,39] When the 2.8 kb-LacZ transgene was expressed in the rbp5 mutant background, there was no effect on expression in the midgut at both the late L3 or early prepupal stages (Figure 6a). Similar results were obtained when the 2.8 kb-LacZ transgene was expressed in a BR-C null (npr1) background in the late L3 midguts. Midguts from later stages of development could not be analyzed because npr1 mutants do not develop beyond this stage. Unlike in the midgut, the expression of the 2.8 kb-LacZ transgene was severely reduced in salivary glands from

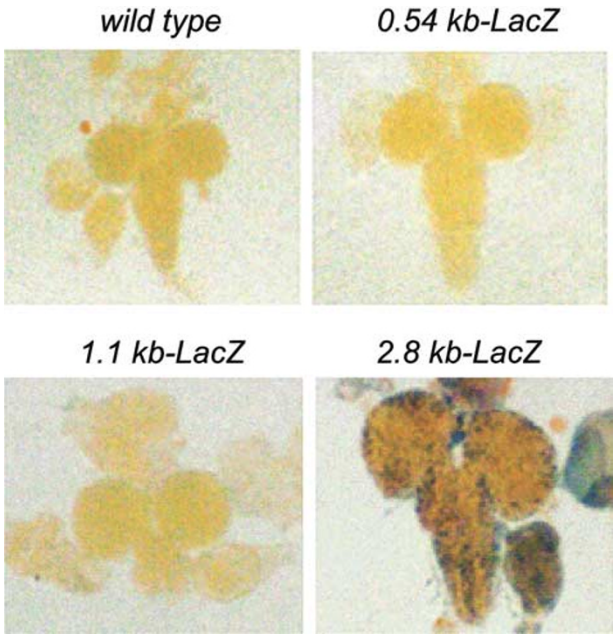


Figure 4 Expression of various *dronc* promoter-*LacZ* transgenes in the larval brain lobes. The brain tissue was dissected out from late L3, and stained with X-gal as in Figure 3

early pupal stages in the rbp5 mutant and late larval stages in the npr1 mutant (Figures 6b, 7a). These data suggest that BR-C is essential for proper expression of *dronc* in salivary glands, but not in the midgut.

The E93 gene encodes a novel nuclear protein, and is specifically required for ecdysone-induced cell death in both the midgut and salivary glands.[19] In E93 null mutants, larval midgut and salivary glands persist into early pupal stages when development is arrested.[19] When the 2.8 kb-LacZ transgene was introduced into the E93 mutant background, the expression of β-gal was strongly suppressed in all regions of the midgut and salivary glands at the time prior to the histolysis of these tissues (Figures 6, 7b). These data indicate that E93 is required for the maximal expression of *dronc* during PCD in both tissues.

We also analyzed the effect of E74A mutants on 2.8 kb-LacZ transgene expression. Consistent with previous findings,[39] we observed no significant qualitative or quantitative changes in X-gal-stained salivary glands (data not shown). Likewise, RT-PCR showed 2.8 kb-LacZ transgene activity in E74A-deficient salivary glands comparable to controls (Figure 7c, top panel). Although the endogenous *dronc* transcript appears to be somewhat reduced in the E74A-deficient salivary glands in Figure 7c (middle panel), this was not always seen. These data suggest that E74A does not play a crucial role in regulating *dronc* expression during salivary gland cell death.

Discussion

DRONC is the only CARD containing apical caspase in *Drosophila* and is predicted to be the main initiator caspase functionally similar to CED-3 in *C. elegans* and caspase-2/caspase-9 in mammals.[23,37] RNAi studies suggest that

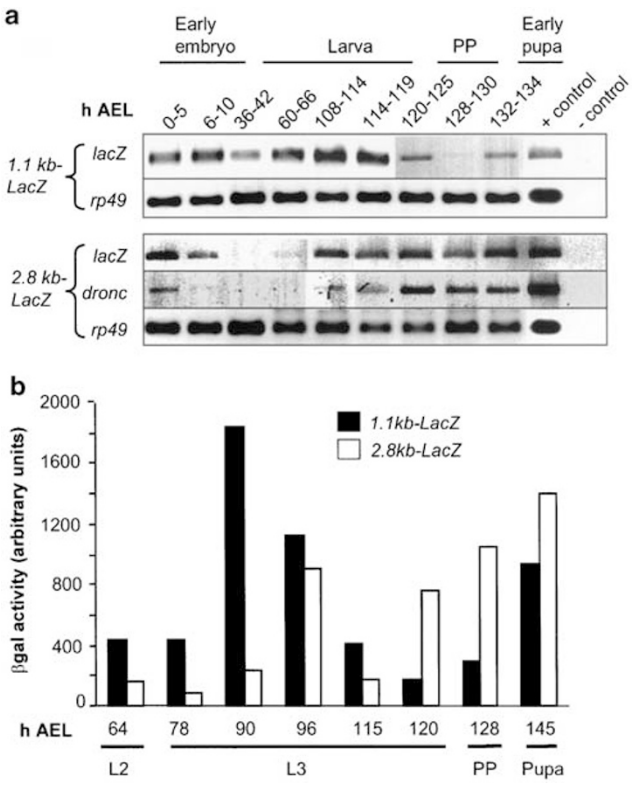


Figure 5 Expression of endogenous *dronc* and the promoter-*LacZ* transgenes in animals at various stages of development. In (**a**), RT-PCR analysis of *LacZ*, *dronc*, and *rp49* (as a control) expression is shown. The developmental stages are indicated as h AEL. Both the 1.1 and 2.8 kb promoter-*LacZ* constructs respond to the late L3 and the prepupal ecdysone pulses, which direct endogenous *dronc* expression. In all, 22–25 cycles of PCR were carried out using cDNA prepared from animals at various stages of development and gene-specific primers. Note that the expression pattern of the 2.8 kb-*LacZ* transgene is similar to that of endogenous *dronc*. However, the expression of the 1.1 kb-*LacZ* transgene is deregulated, and can be clearly seen in the early larval stages when endogenous *dronc* expression is very low. In prepupal (PP) and early pupal stages, the 1.1 kb-*LacZ* transgene expression is lower, presumably due to a lack of expression in salivary glands. No expression for the 0.54 kb-*LacZ* transgene was observed at any stage (data not shown). The positive (plasmid DNA) and negative (−cDNA) PCR controls are shown in the last two lanes of each panel. In (**b**), β-gal activity was determined in the larvae, PP, and pupae from 1.1 and 2.8 kb-*LacZ* transgenic animals. Note that the 1.1 kb promoter-driven *LacZ* expression is much higher in early larval stages. L2, second instar larvae; L3, third instar larvae

DRONC is essential for PCD during embryogenesis[37] and for ecdysone-induced cell death.[38] As evident from this study and previous observations,[23] the expression of *dronc* transcript during development is highly dynamic. As intracellular levels of DRONC protein may determine the sensitivity of cells to undergo PCD, it is important to understand how *dronc* expression is regulated during development. As summarized in Figure 8, the results reported in this paper provide the first analysis of the *dronc* promoter, and form a basis for further studies. Given that *dronc* is regulated both temporally and spatially and *dronc* gene expression, at least during metamorphosis, is regulated by the steroid hormone ecdysone, the *dronc* promoter provides a convenient model system for dissecting out the components of the regulatory apparatus mediating gene expression by nuclear hormones.

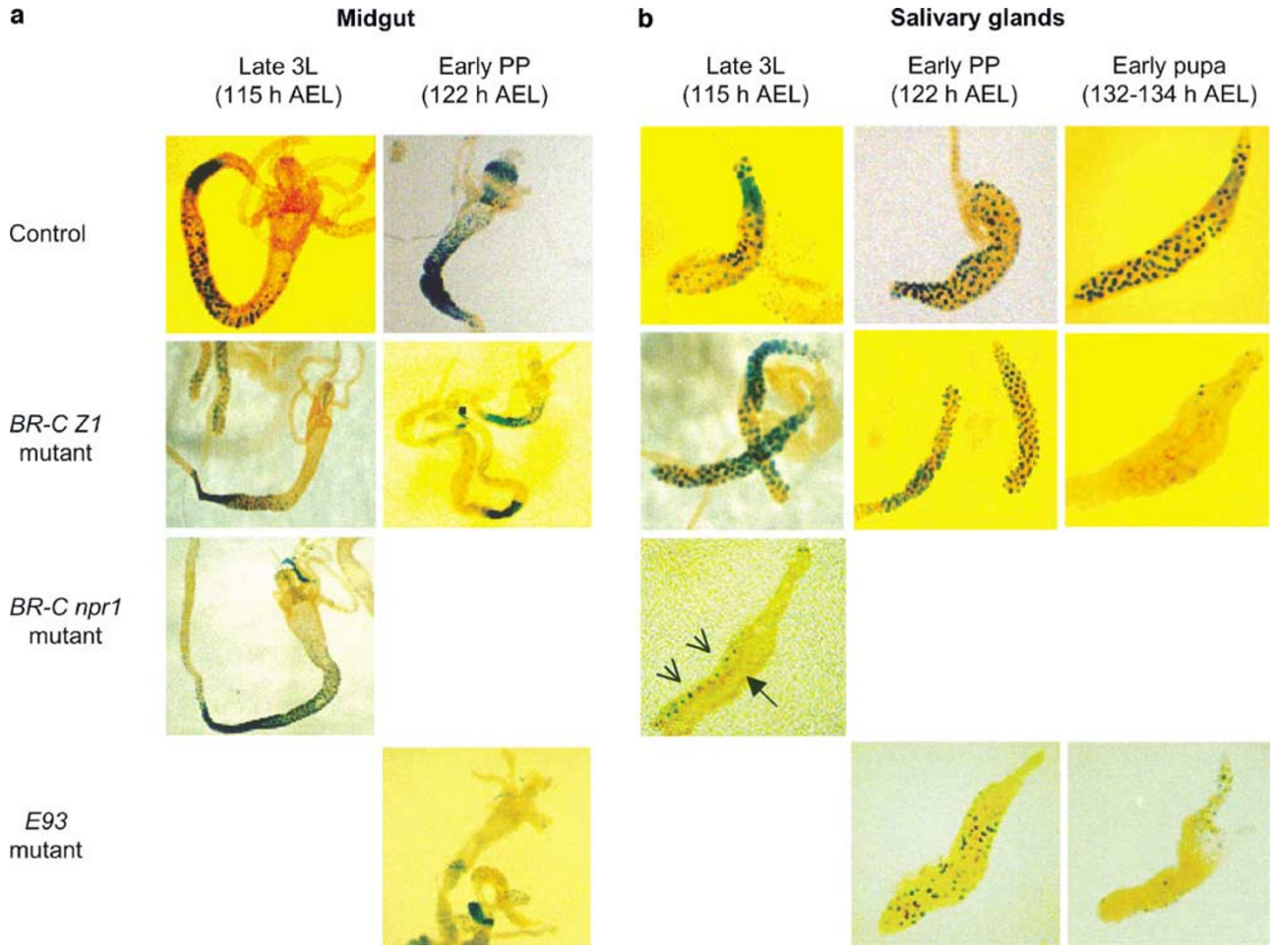


Figure 6 Expression of various *dronc* promoter-*LacZ* transgenes in the midgut (**a**) and salivary glands (**b**), from *BR-C* and *E93* mutants. The 2.8 kb promoter-*LacZ* transgene was expressed in a wild-type (control), *BR-C* (*rbp5* or *npr1*) or *E93* null background, as described in Materials and Methods. The midgut and salivary glands were dissected out from late 3L (~115 h AEL) and early prepupae (PP) (~122 h AEL), and stained with X-gal. Salivary glands were also dissected out from the early pupal stage (132–134 h AEL) animals, and stained with X-gal. β-Gal staining seen in the *BR-C npr1* salivary gland (**b**, late 3L) represents fat body (arrow heads), while the salivary gland (arrow) itself is not stained. In *E93* mutants, staining in both the midgut and salivary glands is reduced, and can only be seen in long exposures (bottom panels in (**a**) and (**b**))

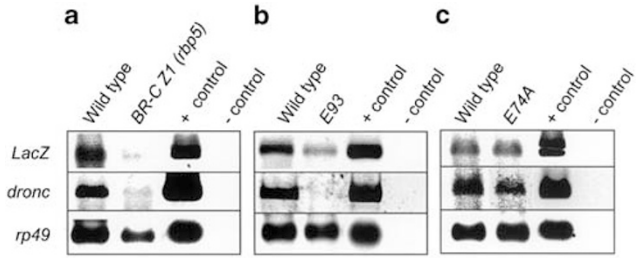
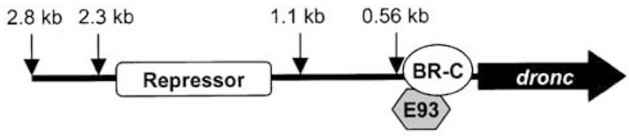


Figure 7 RT-PCR analysis of *LacZ* and *dronc* expression in the salivary glands of 2.8 kb promoter-*LacZ* transgenic flies in *BR-C Z1* (**a**), *E93* (**b**), and *E74A* (**c**) null background. Salivary glands were dissected out from animals around 130–133 h AEL. *Rp49* expression was used as a control. The promoter-*LacZ* transgene (2.8 kb) was in either the wild-type (W^{1118}) or mutant background as indicated. The positive (plasmid DNA) and negative (−cDNA) PCR controls are shown in the last two lanes of each panel



	SG	MG	BL	OV
2.8 kb-LacZ	+	+	+	+
1.1 kb-LacZ	-	+	-	+
0.56 kb-LacZ	-	-	-	-

Figure 8 Summary of the *dronc* promoter analysis. Potential BR-C and E93-binding elements and repressor-binding site are indicated. The lower panel summarizes the results of *dronc* promoter-*LacZ* expression in various tissues. SG, larval or prepupal salivary glands; MG, larval or prepupal midgut; BL, larval brain lobes; OV, adult ovaries

Experiments outlined in this paper demonstrate that 2.3 kb of the *dronc* promoter is largely sufficient for temporal expression (compared to endogenous *dronc*) throughout development. Previous experiments have shown that *dronc* is predominantly expressed in the larval and prepupal salivary glands and midgut, and larval brain lobes.[23] We have shown that 2.3 kb of the *dronc* promoter contains all necessary elements for correct spatial regulation of *dronc* expression in these tissues.

In order to identify transcription factors responsible for both temporal and spatial regulation of *dronc* and ecdysone-mediated PCD, it is of vital importance to elucidate the regions of the promoter essential for *dronc* expression in different tissues. In addition, it would be of interest to determine if there is a single promoter region controlling the spatial expression profile of *dronc*, or if different promoter regions are required in different tissues. *LacZ* transgenic reporter experiments reveal that the 2.3 kb promoter is the minimal requirement for correct expression in brain lobes and salivary glands. Furthermore, the region between 1.1 and 2.3 kb contains transcription factor-binding sites essential for expression in these tissues. This region also seems to harbor a repressor element important to keep *dronc* levels low during periods when ecdysone titers are low. Surprisingly, regulation of *dronc* transcription is markedly different in the midgut. The region between 1.1 and 2.3 kb is not important for transcription in this tissue, as 1.1 kb of the promoter is sufficient for expression. These results clearly demonstrate that distinct regions of the promoter are required for expression in different tissues, and implies that different transcription factors regulate *dronc* expression in a tissue-dependent manner.

The two ecdysone-induced transcription factors BR-C and E93 are essential for *dronc* expression in salivary glands.[1,12,19,39] In the midgut, however, only E93 seems to be important.[40] Our results of *dronc* promoter-*LacZ* transgenic expression in flies deficient in BR-C and E93 are consistent with recent findings.[19,38,39,40] *LacZ* expression driven by the 2.8 kb promoter is severely impaired in salivary glands of *BR-C* (*rbp5* and *npr*) or *E93* mutants, whereas expression is impaired only in the midgut of *E93* mutant background animals. This further supports the idea that the mechanisms governing *dronc* regulation are tissue specific. The key questions arising from these experiments are: why does the BR-C Z1 isoform (*rbp5* mutant) regulate *dronc* in the salivary glands and not in the midgut? What factors are binding to the 1.1–2.3 kb region of the promoter in salivary glands, and why are they not as important in the midgut? Our previous results show that either BR-C Z1- or BR-C Z1-regulated proteins bind to the *dronc* proximal promoter (7–176 bp) and control its expression.[38] Transactivation of the 2.8 kb promoter by BR-C Z1, however, was only seen in specific cell types.[38] Given that BR-C Z1 is also expressed in the midgut, this implies that it may be acting through cofactors which are not expressed in the midgut, yet are specifically recruited to the *dronc* promoter. Alternatively, BR-C Z1 induces the expression of another factor which binds to the promoter, and this factor is absent in the midgut.

Since we have shown that the proximal promoter alone (0.54 kb) is not sufficient for expression in the salivary gland, we believe that BR-C Z1 (or a Z1-regulated protein) is cooperating with other transcription factors binding upstream (1.1–2.3 kb), which are essential for salivary gland expression. We have found that E93 acts through the first 600 bp of the *dronc* promoter by transactivation studies (DC and SK, unpublished data); however, no direct binding of E93 to the *dronc* (or any other) promoter has been shown so far. Additionally, a preliminary analysis indicates the presence of an EcR/Usp-binding site between 1.1 and 2.3 kb of the *dronc* promoter, and *in vitro* experiments show that this element may be important in regulating *dronc* expression (DC, TD, and SK, unpublished data). Since the proximal promoter (0.54 kb) alone is not sufficient for expression, cooperation of BR-C and E93 with EcR/Usp and other unknown factors may be important for temporal and spatial regulation of *dronc* expression during development. Identification of these factors will be important for fully understanding *dronc* transcription during development.

Overall, we have established the minimal *dronc* promoter requirement for spatial and temporal expression to be within the 2.3 kb region upstream of the *dronc* gene. This region is important for both BR-C- and E93-mediated transcription in salivary glands and E93 transcription in the midgut. Importantly, the 1.1–2.3 kb promoter region harbors elements important for salivary gland expression and a putative repressor element. The 0.54–1.1 kb promoter region is important for expression in the midgut. These regions will form the basis of future experiments designed to identify factors necessary for the regulation of *dronc* expression during PCD.

Materials and Methods

Transgenic and mutant stocks and crosses

All *dronc* promoter-nuclear *LacZ* reporter constructs were generated by double-insert ligations into the pCasper-4 p-element-mediated transformation vector,[41] in the following manner. Variable promoter lengths were PCR amplified from *D. melanogaster* genomic DNA using a common reverse primer containing an *Nco*1 site at the ATG of *dronc*. 5′ blunt and 3′ *Nco*1 *dronc* promoter fragments were ligated with 5′ *Nco*1 and 3′ *Not*1-cut *LacZ* into pCasper-4 with a Klenow end-filled *Eco*R1 and a *Not*1 site.

Transgenic flies were generated and maintained as previously described.[42] Briefly, *dronc* promoter-*LacZ* reporter germline transformation constructs ($\sim$500 ng/μl) and transposase helper plasmid Δ2–3 ($\sim$150 ng/μl) were microinjected into precellularized *Drosophila W*^{1118} embryos. w^{+} transformants were screened by eye color, mapped, and homozygous stocks generated by established techniques.

For *BR-C Z1* isoform mutant (*rbp5*)[11] experiments, males homozygous for the second or third chromosome *dronc* promoter-*LacZ* transgenes were crossed to virgin *yrbp5* heterozygote females balanced with the sex chromosome balancer *Binsn*. The yellow mouthhook male larvae, prepupae, and early prepupae (hemizygous for the mutated *BR-C* gene and heterozygous for the reporter) were selected for analysis. Control flies are male larvae with normal mouthhooks, which have wild-type *BR-C* genetic backgrounds (*Binsn/Y*). A similar crossing strategy was employed to analyze reporter activity in the *BR-C* null mutant *npr1*.[43]

Fly stocks homozygous for a second chromosome 2.8 kb *dronc* promoter-*LacZ* transgene and heterozygous for *TM6B* balancer chromosome, and either the *E93* mutant[19] or deficiency (*Df*[3R]*93F*^{x2}), were crossed, and non-*TM6B* progeny (DrPr2.8kb-*LacZ*; *E93*′/*Df*[3R]*93F*^{x2})

compared to control *TM6B* progeny (DrPr2.8kb-*LacZ*; *E93*[1] or *Df*[3R]*93F*[x2]/*TM6B*). A similar crossing strategy was undertaken for DrPr2.8kb-*LacZ*, *E74A* mutant analysis, employing the mutant *E74A*[Pneo16] and *E74A* deficiency (*Df*[3L]*st-8IK19*).

Staging of animals

Developmental stages from the embryos to L3 stages were acquired by ageing animals from the time of egg deposition on grape agar plates to the desired stages at 25°C. L3 stages were determined by the gut-clearance technique following the growth of animals on bromophenol blue supplemented food.[44] Prepupal and pupal stages were attained by collecting newly pupariated animals from clear-gutted L3 populations every 30 min, and ageing at 25°C to desired stages before collection and analysis.

For mutant and *dronc* promoter–reporter crosses, approximately 50 virgins and males were left for 3 days in vials containing standard cornmeal media, and then transferred to grape agar lay tubes for staging lays. Animals were collected, rinsed briefly in PBS, frozen in liquid nitrogen, and stored at −70°C for assaying or RNA extraction, or dissected and stained as below.

Detection of β-gal expression

β-Gal detection in embryos was carried out essentially as described.[45] Briefly, the embryos were collected from grape agar lay plates, dechorionated in 50% bleach/50% PBT, washed in water and fixed for 20 min in 50% heptane/50% fixative (0.1 M sodium phosphate, pH 7.5/4% formaldehyde) while nutating. Embryos were rehydrated with 0.7% NaCl/0.04% Triton X-100 for 5 min, stained with X-gal solution (5 mM $K_4[Fe(CN)_6]$/5 mM $K_3[Fe(CN)_6]$/0.2% X-gal stock (20 mg/ml) in PBS), and incubated at 37°C for a period dependent on transgene expression levels (usually overnight). Embryos were devitellinized with 50% heptane/50% methanol by vortexing for 20 s, washed with ethanol and mounted in 80% glycerol, 20% PBT for analysis. Adult, pupal, and larval tissues were stained as described.[45] Briefly, the tissues were dissected in PBS, fixed for 10 min in 2.5% gluteraldehyde/50 mM Pipes (pH 7.5), washed 3 × 5 min in PBS, and incubated at 37°C in X-gal staining solution. Tissues were washed in PBS, incubated overnight in 80% glycerol/20% PBS, mounted and photographed.

Quantitative β-gal assays

Quantitation of β-gal activity was carried out essentially as described,[45] with some modifications. In all, 5–10 animals were homogenized in 100 μl assay buffer (50 mM potassium phosphate/1 mM $MgCl_2$, pH 7.5), the volume made up to 1 ml and vortexed briefly. A volume of 10 μl was transferred to 200 μl of chlorophenol red β-D-galactopyranoside (CPRG) solution (1 mM CPRG final concentration), and incubated at 37°C. β-Gal activity is represented as the change in A^{574} over 2 h, divided by the sample protein concentration, and then multiplied by an arbitrary constant. Control values from W^{1118} samples were subtracted to account for endogenous β-gal activity.

RT-PCR

Total RNA was extracted from the dissected tissues and whole animals using Trizol reagent (Invitrogen), according to the manufacturer's protocol. Up to 5 μg of total RNA was used as template for cDNA synthesis, in a 15 μl reaction with 200 ng of oligo $d(T)_{18}$ primer, using a First-Strand Synthesis Kit (Amersham), according to the manufacturer's protocol. Using 2 μl of cDNA template, PCR amplification was performed using appropriate primers in a 50 μl reaction employing 22–25 cycles. *Drosophila* Rp49 was used as a control. PCR reaction (10 μl) was electrophoresed on a 1.5% agarose gel for analysis.

Acknowledgements

This work was supported by the National Health and Medical Research Council of Australia. TJD was supported by a Dawes Postgraduate Award from the Royal Adelaide Hospital. We are grateful to Eric Baehrecke, Carl Thummel, and *Drosophila* Stock Center for fly stocks.

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