Germline Cell Death Is Inhibited by *P*-Element Insertions Disrupting the *dcp-1/pita* Nested Gene Pair in Drosophila

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Manuscript received May 16, 2003
Accepted for publication August 13, 2003

ABSTRACT

Germline cell death in Drosophila oogenesis is controlled by distinct signals. The death of nurse cells in late oogenesis is developmentally regulated, whereas the death of egg chambers during mid-oogenesis is induced by environmental stress or developmental abnormalities. *P*-element insertions in the caspase gene *dcp-1* disrupt both *dcp-1* and the outlying gene, *pita*, leading to lethality and defective nurse cell death in late oogenesis. By isolating single mutations in the two genes, we have found that the loss of both genes contributes to this ovary phenotype. Mutants of *pita*, which encodes a C_2H_2 zinc-finger protein, are homozygous lethal and show dumpless egg chambers and premature nurse cell death in germline clones. Early nurse cell death is not observed in the *dcp-1/pita* double mutants, suggesting that *dcp-1*$^+$ activity is required for the mid-oogenesis cell death seen in *pita* mutants. *dcp-1* mutants are viable and nurse cell death in late oogenesis occurs normally. However, starvation-induced germline cell death during mid-oogenesis is blocked, leading to a reduction and inappropriate nuclear localization of the active caspase Drice. These findings suggest that the combinatorial loss of *pita* and *dcp-1* leads to the increased survival of abnormal egg chambers in mutants bearing the *P*-element alleles and that *dcp-1* is essential for cell death during mid-oogenesis.

DURING Drosophila oogenesis, oocytes develop within individual cysts of 16 germline cells, surrounded by somatically derived follicle cells (Spradling 1993). The 16 germline cells give rise to a single oocyte and 15 supporting nurse cells, which synthesize proteins and RNA destined for the oocyte. In late oogenesis, the nurse cell cytoplasm is rapidly transported (dumped) into the oocyte as the nurse cells initiate programmed cell death (stages 11–13; Foley and Cooley 1998; McCall and Steller 1998). Programmed cell death can also occur during mid-oogenesis (stages 7 and 8) in response to nutrient deprivation, developmental abnormalities, or other insults (Giorgi and Deri 1976; Chao and Nagoshi 1999; De Lorenzo *et al.* 1999; Nezis *et al.* 2000; Drummond-Barbosa and Spradling 2001). These insults lead to the degeneration of the entire egg chamber, both the germline and somatic follicle cells. Cell death in mid-oogenesis is thought to be the outcome of a checkpoint where the state of the egg chambers is monitored before making the investment of vitellogenesis (Giorgi and Deri 1976; Chao and Nagoshi 1999; Buszczak and Cooley 2000). This checkpoint may be controlled by signaling through the steroid hormone ecdysone (Buszczak *et al.* 1999; Carney and Bender 2000).

The mechanism of cell death in the fly ovary is not well understood. Several components of the apoptotic machinery are expressed during oogenesis, but it is unknown which ones are required (Buszczak and Cooley 2000). The major effectors of apoptotic cell death are caspases, a family of aspartyl proteases (reviewed in Earnshaw *et al.* 1999; Nicholson 1999; Shi 2002). Caspases fall into two classes, the initiators, which interact with upstream adaptor proteins and function by cleaving other caspases, and the effectors, which cleave cellular proteins leading to the morphological changes observed in dying cells. Seven caspases have been reported in Drosophila, three in the initiator class and four in the effector class (Kumar and Doumanis 2000). One of the effector caspases, Drice, is activated to high levels in egg chambers that degenerate during mid-oogenesis in response to nutrient deprivation, but only to moderate levels during developmentally regulated nurse cell death in late oogenesis (Peterson *et al.* 2003). These variations in caspase activity as well as morphological differences suggest that different cell death mechanisms may operate during mid- and late oogenesis.

The caspase Dcp-1 was shown to play a role in nurse cell death in late oogenesis on the basis of the phenotypes caused by single *P* elements inserted in the *dcp-1* 5′-untranslated region (UTR; McCall and Steller 1998). Annotation of the Drosophila genome (Adams *et al.* 2000) predicts that the *dcp-1* gene is nested within an intron of the *CG3941* (*pita*) gene, encoding a member of the C_2H_2 zinc-finger protein family. In this report,

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we show that *P* elements inserted in the *dcp-1* gene disrupt expression of both *dcp-1* and the outlying gene *pita*. By isolating separate mutations in *dcp-1* and *pita* and by carrying out a series of rescue experiments, we have determined that *pita* function is required for proper egg chamber development and that *dcp-1* is essential for germline cell death during mid-oogenesis. The combinatorial loss of *dcp-1* and *pita* leads to defective nurse cell death and abnormal egg chambers. Loss of *dcp-1* alone inhibits germline cell death at the mid-oogenesis checkpoint but does not inhibit developmentally regulated nurse cell death in late oogenesis, suggesting that these two types of germline cell death utilize distinct components of the cell death machinery.

MATERIALS AND METHODS

Drosophila stocks: The *P*-element alleles were obtained from the Bloomington Stock Center and the Berkeley *Drosophila* Genome Project (BDGP). Germline clone (GLC) analysis was carried out as previously described using the *FLP/FRT/ovo*^{D} system (Chou and Perrimon 1996; McCall and Steller 1998). *Df(2R)bw*^{PRs}*/SM6* flies were obtained from Bruce Reed, *Sco/SM1* and *cn bw sp* flies from Terry Orr-Weaver, and *nanos-GAL4VP16* flies from Pernille Rorth. The *BB127* enhancer trap, *CyO, Kr-GAL4 UASGFP* (*CyO, GFP*) balancer, and all other strains were obtained from the Bloomington Stock Center.

Mutagenesis screens: Isogenic *cn bw sp* males were mutagenized with 35 mm EMS in 10% sucrose overnight and 54 lethal mutations that failed to complement *Df(2R)bw*^{PRs}*/SM6* were recovered from 1700 fertile F_1 males. Three of these mutations failed to complement *PZ08859* and were analyzed further. To generate *dcp-1* mutants, *P*-element reversion was carried out using *y w; k05606, w*$^{+}$*/CyO* flies crossed to *y w; Sco/CyO; Sb* Δ*2-3/TM6. white* non-*Sco* progeny were collected as heterozygous *k05606* revertants (*dcp-1*^{Prev}) and screened by PCR for small insertions or deletions.

Rescue constructs: The *pita* cDNA was obtained as clone LD15650 from Invitrogen (San Diego) and subcloned into *pCaSpeR-hs* (Thummel and Pirrotta 1992) to generate *HS-pita*. A 4.4-kb genomic fragment, corresponding to −1769 to +2578 relative to start of the *dcp-1* cDNA, was generated by PCR from genomic P1 clone DS07147 (provided by the BDGP) and cloned into *pCaSpeR4* (Thummel and Pirrotta 1992) to generate *pCaSpeR-4.4dcp-1*. Transgenic flies were generated by standard procedures.

Molecular analysis: The sites of *P*-element insertion were determined by PCR and DNA sequencing as described (Song *et al.* 1997). The EMS-induced alleles were balanced with *CyO, GFP*, and homozygous third instar larvae that lacked green fluorescent protein (GFP) expression and had melanotic tumors were selected for PCR and DNA sequencing. PCR of *k05606* revertants was carried out with primers that amplify a 317-bp fragment spanning the *k05606* insertion site. RT-PCR was carried out on total RNA from *y w* control or homozygous non-GFP mutant embryos. Primers corresponding to the 3′ ends of *nuclear lamin Dm*$_0$ and *pita* were used in the reverse transcription reaction at final concentrations of 4×10^{-7} pmol/μl and 5×10^{-2} pmol/μl, respectively. Primers used for RT-PCR flanked an intron so the product could be distinguished from amplification of genomic DNA.

Generation of antisera and Western analysis: The Dcp-1 peptide antibody was generated against the C terminus of Dcp-1 (sequence DKPNGNKAG) in rabbits and affinity purified by Zymed Laboratories (South San Francisco, CA). Homogenized embryos were analyzed by 10% SDS-PAGE, followed by immunoblotting with the Dcp-1 antibody diluted 1:1000 or with an anti-Armadillo monoclonal antibody supernatant diluted 1:100 (anti-Armadillo developed by E. Wieschaus and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, Iowa).

Staining procedures: Ovaries from flies conditioned on wet yeast paste or nutrient deprived (Peterson *et al.* 2003) were dissected and stained as described (Verheyen and Cooley 1994) except that stained tissues were mounted in Vectashield with or without 4′,6-diamidino-2-phenylindole (DAPI) to label nuclei (Vector Labs, Burlingame, CA). For nuclear analysis, fixed ovaries were incubated in RNase A (20 μg/ml) for 30 min, washed in phosphate-buffered saline + 0.1% Triton-X, and mounted in Vectashield with propidium iodide (Vector Labs). Anti-CM-1 (Srinivasan *et al.* 1998) was diluted 1:1500 and anti-active Drice (Yoo *et al.* 2002) was diluted 1:1000, followed by goat-anti-rabbit-Cy-3 at 1:200 (Jackson ImmunoResearch Labs, West Grove, PA). Controls where the primary antibody was excluded failed to show any staining. β-Galactosidase detection was carried out as described (McCall and Steller 1998). Samples were viewed on an Olympus BX60 and photographs were taken with film or an Olympus Magna Fire SP digital camera. Confocal images were taken on an Olympus Fluoview confocal microscope. All images were processed in Adobe Photoshop.

Computational methods: Nested gene pairs were determined using genome annotation files from release 3.0 of the Drosophila genome (Misra *et al.* 2003). The introns from the assembled gene pair data set were aligned with the *P*-element insertion site sequences from the BDGP using BLASTN (Altschul *et al.* 1997) with an *E*-value threshold of 1e-15. The cytological location of each *P* element was verified with data found in http://www.flybase.org.

RESULTS

***P*-element insertions in *dcp-1* affect a flanking gene:** The insertion sites have been determined for four *P* elements within the *dcp-1* gene, *PZ01862, PZ02132, PZ08859*, and *k05606* (Figure 1A; Song *et al.* 1997). Three are located within the 5′-UTR of the gene, and one is located within the coding region of the first exon. All four *P*-element alleles show similar phenotypes as reported previously: larval lethality as well as abnormal nurse cell death in germline clones (Song *et al.* 1997; McCall and Steller 1998). None of the *P*-element alleles show detectable Dcp-1 protein by Western blot (Figure 1B), suggesting that they are strong loss-of-function or null alleles.

The sequencing and annotation of the Drosophila genome have revealed that *dcp-1* is nested within an intron of another gene, *CG3941* or *pita* (Adams *et al.* 2000). To determine if the *dcp-1 P*-element alleles could be affecting proper transcription or splicing of *pita*, we examined *pita* transcript levels by RT-PCR. Indeed, *pita* mRNA was absent from the *dcp-1 P*-element strains (Figure 1C). Thus, the *dcp-1 P*-element insertions impair the expression of both *dcp-1* and *pita*.

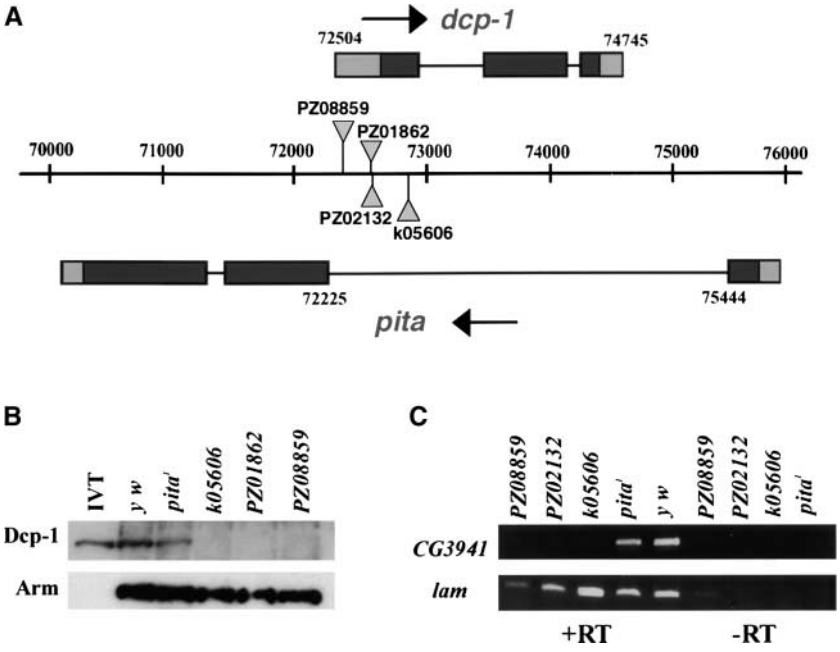

FIGURE 1.—Molecular analysis of *pita* alleles. (A) Map indicating the locations of *CG3941* (pita) and *dcp-1* relative to the chromosome. Numbers are taken from genomic scaffold sequence, accession no. AE003461. Lightly shaded boxes represent the 5′- and 3′-UTRs and lines represent intronic regions. *P*-element insertion sites for *PZ08859*, *PZ01862*, *PZ02132*, and *k05606* are located at 72610, 72733, 72893, and 73012, respectively. (B) Western analysis of mutant lines. Equal amounts of protein from *y w* control or homozygous mutant 10- to 13-hr-old embryos were analyzed by SDS-PAGE and immunoblotted with anti-Dcp-1 or anti-Armadillo antibodies. *In vitro* translated Dcp-1 (IVT) is shown as a size reference. (C) RT-PCR analysis of mutant lines. Equal amounts of total RNA were analyzed using primers that amplify *CG3941* or nuclear lamin Dm_0 as a control.

Point mutations in *pita* cause larval lethal phenotypes similar to the *P*-element alleles: To investigate which gene was responsible for the phenotypes observed in the *P*-element mutants, an EMS mutagenesis was performed to isolate noncomplementing point mutations. Three homozygous lethal EMS-induced alleles that failed to complement the *P*-element alleles were identified. Surprisingly, all three EMS-induced alleles had mutations in *pita* and not in *dcp-1*, suggesting that the larval lethal phenotype previously attributed to *dcp-1* is due to loss of *pita*.

pita encodes a 683-amino-acid protein composed of 10 C_2H_2 Zn fingers with a potential acidic transactivation domain (MITCHELL and TJIAN 1989) in the C terminus (Figure 2). The *pita*^{1} (H472Y) and *pita*^{2} (H448Y) alleles are predicted to alter histidine residues necessary for Zn binding (MILLER *et al.* 1985). *pita*^{3} is a nonsense mutation (Q315amber) predicted to truncate the protein after the first Zn finger. On the basis of this molecular evidence as well as genetic evidence (not shown), we believe that *pita*^{3} is a null mutation.

The *pita*^{3} allele had a homozygous larval lethal phenotype similar to the *P*-element alleles, including melanotic tumors, underdeveloped imaginal discs, and tracheal defects (data not shown). *pita*^{1} mutants also died as larvae with melanotic tumors; however, the imaginal discs appeared normal. The *pita*^{2} allele had a weaker phenotype with lethality occurring at the prepupal stage. The weaker phenotype seen in *pita*^{2} compared to *pita*^{1} suggests different requirements for individual Zn fingers or a partial loss of function when the last histidine residue is altered within the finger (WOLFE *et al.* 2000).

***pita* germline clones display premature nurse cell death unlike the *P*-element alleles:** All four *P*-element mutants showed abnormal oogenesis in GLCs, and the strength of the phenotype varied depending on the allele. Nuclear β-galactosidase (β-gal) was used to visualize the breakdown of nurse cell nuclei in late oogenesis (COOLEY *et al.*

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  1 MAAKLEVREA MLTEKRVCRF CLTEQKLASI FEENPRVKTT
 41 ANLPLQIMAI TAIEVYAGDG MPGHICLECR LLFEHCYRFK
 81 QMCKRAETLL RQYPLTGNWP SPLEKPRAPM TMVASKKLLV
121 VPAKTAEPSE TPKKLLNTMA KSSSQVIIED VQVLESAMVT
161 PRTVAGSSPV PRRSHAYELK VDNNQELSMD DVQSMLEDMA
201 SELEKEFPDI PQKASPVKPK VLNKSSIRIL NKGPAAPVEP
241 RLATPKVKRD DSGNVAIVTE VLDSDLPLDD QDDPTKNAEK
281 VATDVFPCPD CERSFPLQQL LEIHRLNHTR SRSHQCLLCE
321 KSFFSKYDLA KHNFVHTGER PFKCAICSKA FTRKALLHRH
361 ERTHTDVPKF ICVYCEKPFL SRQEMEKHAE RHCKKRPFQC
401 GVCTKSFAFK QGLERHETVH STNLPFPCQH CERSFSTASK
441 LARHLVAHAG KRAYPCKYCH KSYMLSHHLS RHLRTHTQTS
481 DASFVCSECK VSYSNYNDLL DHALIHATAS LKCPMCRKQI
521 EDIDSVESHM DQHKQSERHA CEFCDHIFLT QKCLQRHIED
561 DHVVEMEPYQ NEFEDDGEGE GGVDEKEEHL DDFEDMDNVK
601 QEEFVTEYLE DDALYEDHLD DSDESFTPPP PKQRKTNPPK
641 DAQQSVRQTR SRDAQRITQK TGKNEGKPHH KLERNLKNRR SAK
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FIGURE 2.—Pita sequence and mutations. Pita is a 683-amino-acid protein, containing eight Zn fingers that strongly follow the consensus for C_2H_2 Zn-finger motif (underlined) and two additional regions that fit a looser consensus (dotted line). Pita has the Krüppel-related H-C linker sequence HTGEKPYK between zinc fingers 2 and 3. The zinc-finger region is followed by an acidic domain that may serve as a transcriptional activation domain. There are two possible unipartite nuclear localization signals (boxed, amino acids 394–397 and 631–635). The three EMS alleles all carry mutations within the Zn-finger region (boxed single amino acids). *pita*^{3}, Q315amber, is located just within the second Zn finger. The *pita*^{2} mutation, H448Y, and the *pita*^{1} mutation, H472Y, disrupt histidines in the sixth and seventh Zn fingers, respectively.

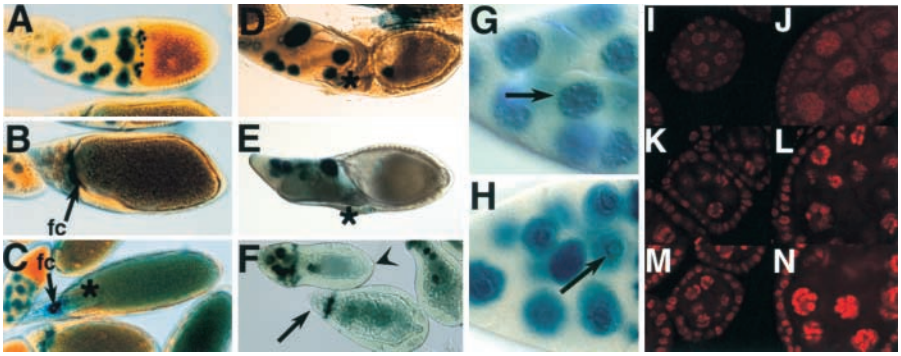

FIGURE 3.—Abnormal oogenesis in *pita* GLCs. (A–C) Wild-type *BB127* enhancer-trap egg chambers stained with X-gal. (A) In stage 10, β-gal is localized to nurse cell nuclei and a subset of follicle cells near the oocyte border. (B) By stage 12, the nurse cell nuclei have released β-gal and it has been transported to the oocyte. fc indicates follicle cells that continue to stain. (C) In stage 14, dorsal appendages are apparent (asterisk) and nurse cells are gone. (D) *PZ08859* GLC stage 14 egg chamber. X-gal reflects *lacZ* expressed from the *PZ08859 P* element. Dorsal appendages (asterisk) are apparent but the egg chamber is dumpless and nurse cell nuclei retain β-gal. (E) *BB127/+; pita*^{1} GLC stage 14 egg chamber shows dumpless phenotype similar to *PZ08859*. Asterisk indicates dorsal appendages. (F) *BB127/+; pita*^{3} GLC egg chambers are severely abnormal, often with thick follicle cells (arrowhead) and/or lacking nurse cell nuclei (arrow). (G) *BB127* (wild-type) stage 10 nurse cell nuclei show a granular morphology (arrow). (H) *pita*^{1} GLC stage 10 nurse cell nuclei show crater-like structures (arrow). A similar morphology is seen in the *P*-element alleles. (I–N) Confocal images of egg chambers stained with propidium iodide to label nuclei. (I) Control stage 5 egg chamber shows discrete chromosomes, which become diffuse by stage 7 (J). (K and L) *PZ08859* egg chambers show discrete chromosomes at early and later stages. (M and N) *pita*^{1} egg chambers show a similar morphology. Magnification: A–F, ×200; G and H, ×400; and I–N, ×600.

1992). In wild-type nurse cells, nuclear β-gal diffused into the cytoplasm beginning in stage 10, before nurse cell dumping occurred (Figure 3, A–C). The *PZ01862* and *PZ02132* GLCs have a variable dumpless phenotype with persisting β-gal-positive nurse cell nuclei (McCALL and STELLER 1998) and a similar moderate phenotype was seen in *k05606* GLCs (data not shown). However, the ovarian phenotype of *PZ08859* was significantly stronger, with ~95% of late egg chambers displaying a strong dumpless phenotype (Figure 3D, $n = 200$). In addition, egg chambers frequently displayed abnormalities at earlier stages, including reduced size, an unusually thick follicle cell layer, and abnormal nurse cell nuclear morphology.

The EMS alleles displayed a range of GLC phenotypes, with some similarities but also distinct differences compared to the *P*-element alleles. The *pita*^{1} allele had a moderate dumpless phenotype, with 80% dumpless stage 14 egg chambers (Figure 3E, $n = 460$). However, *pita*^{1} GLCs had notably fewer stage 14 egg chambers (44%, $n = 1000$) than the *P*-element alleles had (60%, $n = 335$, for the strongest allele, *PZ08859*), suggesting that many of the *pita*^{1} egg chambers degenerated before reaching late oogenesis. *pita*^{3} had a stronger phenotype than *pita*^{1} or *PZ08859*, with many abnormal early egg chambers (Figure 3F). Furthermore, early egg chambers (stages 6–9) from both the *pita*^{1} and *pita*^{3} alleles often displayed a "bowling pin" shape, lacking nurse cell nuclei [seen in 25% ($n = 420$) of *pita*^{1} and 56% ($n = 181$) of *pita*^{3} egg chambers (stages 6–9), as shown in Figure 3F]. This phenotype was not seen in any of the *P*-element alleles. The GLC phenotype of the *pita*^{2} allele was much weaker than the other alleles and although the flies were largely infertile, the majority of egg chambers appeared wild type (data not shown). Thus, the EMS alleles showed variability in phenotypes, with dumpless egg chambers and abnormal nurse cell nuclear morphology like the *P*-element alleles. However, the stronger EMS alleles also showed significant levels of premature nurse cell death. This premature nurse cell death was not observed in the *dcp-1/pita* double mutants, suggesting that *dcp-1*$^{+}$ activity was required for the mid-oogenesis cell death seen in *pita* mutants.

The *P*-element and EMS alleles showed altered nurse cell nuclear morphology (Figure 3, G and H). To investigate the nuclear organization further, we examined egg chambers stained with propidium iodide. Early stage wild-type nurse cell chromosomes are polytene and appear as discrete "blobs" until stage 5, after which the chromosomes disperse, giving the nuclei a diffuse appearance (DEJ and SPRADLING 1999; Figure 3, I and J). However, *PZ08859* and *pita*^{1} GLCs showed persistent individualized chromosome blobs through late stages (Figure 3, K–N). Thus, *pita* may play a role in chromosome dispersal that normally occurs during stage 5.

Expression of *pita* rescues the larval and ovary phenotypes of the *P*-element and EMS alleles: To further confirm that the observed phenotypes were due to *pita*, we performed rescue experiments of the *P*-element and EMS alleles. The *pita* cDNA was expressed under the control of a heat-shock-inducible promoter (*HS-pita*) and 1-hr heat shocks were performed daily during larval and pupal development. Homozygous *PZ02132*, *PZ08859*, *pita*^{1}, or *pita*^{3} flies carrying the *HS-pita* transgene survived to adulthood and appeared normal. In contrast, we were unable to rescue the lethality of the *P*-element alleles by expression of *dcp-1*, using *HS-dcp-1*, *UASp-truncated-dcp-1* (PETERSON *et al.* 2003), or *pCaSpeR-4.4dcp-1* (see MATERIALS AND METHODS; data not shown). Thus, expression of *pita* but not of *dcp-1* could rescue the larval lethality, as well as the imaginal disc and melanotic tumor phenotypes of the different alleles.

The *HS-pita* transgene was also sufficient to rescue the ovary phenotype of the mutants. Homozygous *PZ02132*

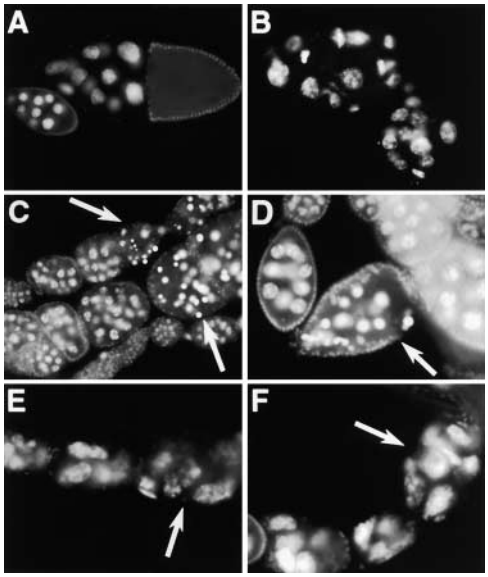

FIGURE 4.—Mutations in *dcp-1* show defective germline apoptosis. Egg chambers were fixed and stained with DAPI to label nuclei. (A–C) Egg chambers from homozygous *dcp-1* and/or *pita* flies rescued from lethality by the *HS-pita* transgene. (A) Egg chambers from *PZ02132/PZ02132* flies 1 day post-heat shock appear normal. (B) Egg chambers from *PZ02132/PZ02132* flies 6 days post-heat shock show a loss of follicle cells and a persistence of nurse cells. (C) Egg chambers from $pita^{1}/pita^{1}$ flies 6 days post-heat shock show egg chambers with degenerating follicle cells and nurse cells (arrows). (D–F) Flies were subjected to nutrient deprivation. (D) Control flies show degenerating egg chambers during mid-oogenesis (arrow). (E and F) Egg chambers from nutrient-deprived $dcp\text{-}1^{Prev1}/dcp\text{-}1^{Prev1}$ flies show a disappearance of follicle cells during mid-oogenesis but the nurse cell nuclei persist (arrows). Magnification: A–F, ×200; D–F were enlarged and are shown at twice the magnification of A–C.

females that reached adulthood following larval and pupal expression of *HS-pita* were initially fertile and showed normal oogenesis (Figure 4A). However, aged flies that were no longer subjected to expression of *HS-pita* showed egg chambers with follicle cell defects. Flies >3 days post-heat shock showed an accumulation of abnormal egg chambers, with relatively normal nurse cells but very few surrounding follicle cells (Figure 4B). Similarly, homozygous *PZ08859* flies rescued with *HS-pita* showed normal oogenesis in young flies and had egg chambers that lacked follicle cells in older flies. However, rescued *PZ08859* flies were sickly and infertile. Thus, the *pita* transgene rescued the viability and ovary phenotype of the *dcp-1/pita* mutants but defects arose several days post-heat shock, suggesting that continued expression of *pita* was necessary for normal oogenesis.

To determine whether the abnormal egg chambers were caused by the loss of *dcp-1* or *pita*, we examined the ovaries of rescued $pita^{1}$ and $pita^{3}$ flies. As seen with the *PZ02132* allele, $pita^{1}$ and $pita^{3}$ flies were initially fertile, but became sterile a few days post-heat shock, suggesting that fertility was dependent on *pita* expression. However, flies that were aged beyond 3 days showed egg chambers with degenerating germline and follicle cells (Figure 4C), rather than the selective follicle cell death seen in the double mutants. These results suggest that *pita* function is required for the survival of follicle cells. The germline cannot normally survive when follicle cells are defective or dying (CHAO and NAGOSHI 1999), and as such the germline cells also died in the *pita* mutant (Figure 4C). However, the germline survived in the *pita/dcp-1* double mutants (Figure 4B), suggesting that *dcp-1* function is required for the death of the germline at the mid-oogenesis checkpoint.

Mutations in *dcp-1* alone lead to defects in germline cell death in mid-oogenesis: To isolate mutations that disrupted *dcp-1* and not *pita*, we used imprecise *P*-element excision of the *k05606* insertion, located within the coding region of *dcp-1*. Several lines that had small insertions consisting of 40 bp of partial *P*-element sequence and the target site duplication were obtained. DNA sequencing confirmed the size of the insert, which would be expected to cause a frameshift, and also revealed an in-frame stop codon within the 40-bp insertion. As expected, these alleles failed to show any Dcp-1 protein by Western blot, but did show normal *pita* expression by RT-PCR (data not shown).

Flies carrying the 40-bp insertion, referred to as the $dcp\text{-}1^{Prev1}$ allele, were homozygous viable and fertile. $dcp\text{-}1^{Prev1}$ ovaries were largely normal but showed occasional egg chambers lacking follicle cells, suggesting that sporadic germline cell death during mid-oogenesis was disrupted. To increase the number of egg chambers dying in mid-oogenesis, flies were nutrient deprived (DRUMMOND-BARBOSA and SPRADLING 2001; PETERSON *et al.* 2003). While *y w* control nutrient-deprived flies occasionally showed egg chambers degenerating during mid-oogenesis (Figure 4D), the $dcp\text{-}1^{Prev1}$ flies showed an accumulation of a large number of egg chambers that lacked follicle cells and had persisting nurse cells (Figure 4, E and F). This phenotype was rescued by *pCaSpeR-4.4dcp-1*, a genomic fragment that includes *dcp-1* but lacks *pita* (data not shown). These findings indicate that *dcp-1* plays an essential role during germline cell death in mid-oogenesis but is not required for normal nurse cell death in late oogenesis or follicle cell death during mid-oogenesis.

Activated caspases are mislocalized in *dcp-1* but not in *pita* mutants: In wild-type ovaries, caspase activity can be detected in nurse cells during stages 10–13 (PETERSON *et al.* 2003) with the CM1 antibody that recognizes the activated form of the effector caspase Drice (YU *et al.* 2002; ARAMA *et al.* 2003). Dcp-1 has been shown to activate Drice *in vitro* (SONG *et al.* 2000). To determine if Dcp-1 was necessary for Drice activation *in vivo*, we compared CM1 staining in wild-type and *dcp-1/pita* mutant ovaries. In wild-type egg chambers, diffuse staining was seen in nurse cell cytoplasm in early and mid-oogenesis (Figure 5A) and accumulated in cytoplasmic aggre-

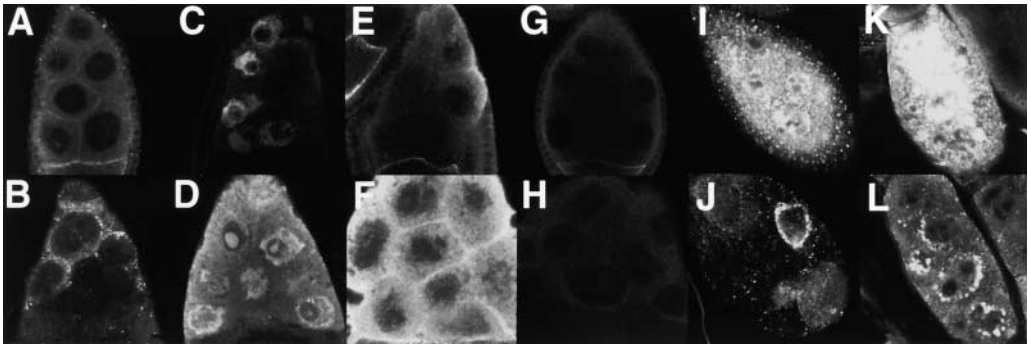

FIGURE 5.—Abnormal caspase localization in *dcp-1/pita* GLCs. Staged egg chambers were immunolabeled with CM1 (A–J) or active-Drice (K and L) antibodies and viewed with confocal microscopy. (A) Wild-type stage 9 egg chamber shows diffuse staining in nurse cell cytoplasm. (B) Wild-type stage 12 egg chamber shows aggregates of bright staining in nurse cell cytoplasm. (C) *PZ08859* GLC stage 9 egg chamber shows nuclear staining and very little cytoplasmic staining in nurse cells. (D) *PZ08859* GLC stage 12 egg chamber shows nuclear staining as well as cytoplasmic staining in nurse cells. (E) $pita^{1}$ GLC stage 9 egg chamber shows diffuse cytoplasmic staining in nurse cells. (F) $pita^{1}$ GLC stage 12 egg chamber shows intense cytoplasmic staining in nurse cells. (G and H) $dcp\text{-}1^{Prev1}$ healthy stage 8 and stage 12 egg chambers show cytoplasmic staining similar to wild type. (I–L) Egg chambers from females subjected to nutrient deprivation. (I) Wild-type stage 8 dying egg chamber shows intense CM1 staining throughout the egg chamber. (J) $dcp\text{-}1^{Prev1}$ stage 8 mutant chamber that lacks follicle cells shows less CM1 staining than in I, and it is concentrated around some of the nurse cell nuclei. (K) Wild-type dying stage 8 egg chamber shows ubiquitous Drice activity. (L) $dcp\text{-}1^{Prev1}$ stage 8 mutant egg chamber shows localized Drice activity similar to CM1. All images were taken at identical confocal settings and at ×400 magnification.

gates in stages 10–13 (Figure 5B; PETERSON *et al.* 2003). In *dcp-1/pita* GLCs (*PZ08859*), CM1 staining was localized inappropriately to nurse cell nuclei beginning in stage 9 (Figure 5C). By stage 12, diffuse staining was also apparent in the nurse cell cytoplasm, but staining was more intense within the nuclei (Figure 5D). Similar mislocalization was seen in *dcp-1/pita* GLCs stained with another antibody generated against active caspase-3 (data not shown). In *pita* GLCs, there was a high level of CM1 staining but it was cytoplasmic as in wild type (Figure 5, E and F). These findings suggest that *dcp-1* activity is required for the proper subcellular localization of active caspases.

$dcp\text{-}1^{Prev1}$ flies did not show a difference in CM1 staining during normal development, suggesting that activation of Drice was not dependent on Dcp-1 during late oogenesis (Figure 5, G and H). However, a significant alteration in CM1 staining was seen in egg chambers degenerating during mid-oogenesis as compared to wild type. While degenerating egg chambers from wild-type nutrient-deprived flies displayed very high levels of CM1 staining (Figure 5I; PETERSON *et al.* 2003), egg chambers lacking follicle cells from starved $dcp\text{-}1^{Prev1}$ flies showed a dramatic decrease in CM1 compared to wild type (Figure 5J). Furthermore, CM1 staining was inappropriately localized to the nuclear lamina of one or a few of the nurse cells (Figure 5J; data not shown). Similar mislocalization was seen in $dcp\text{-}1^{Prev1}$ egg chambers stained with anti-active Drice or another antibody raised against active caspase-3 (Figure 5, K and L; data not shown). However, nuclear staining was not apparent in *PZ08859* GLC egg chambers stained with the anti-Drice antibody (data not shown), suggesting that CM1 may recognize other Drosophila caspases in addition to Drice or that the two antibodies may recognize distinct conformations of Drice. Our findings suggest that Dcp-1 is required for normal levels of Drice activation as well as for its proper localization during mid-oogenesis.

DISCUSSION

The programmed cell death of nurse cells normally occurs late in Drosophila oogenesis, whereas cell death in response to environmental signals occurs during early or mid-oogenesis. *P*-element insertions that disrupt the *pita/dcp-1* nested gene pair show an apparent defect in late nurse cell death. By isolating single mutations in each of the genes, we have determined that the loss of both genes is likely to contribute to this phenotype.

pita loss of function causes developmental abnormalities during oogenesis, perhaps as a failure of Pita to affect transcription, as has been reported for other C_2H_2 Zn finger proteins (WOLFE *et al.* 2000). Pita may specifically affect genes required for development or cell cycle regulation or may act more generally on a large number of target genes. The developmental abnormalities caused by loss of *pita* may trigger the initiation of cell death in mid-oogenesis, leading to the activation of Dcp-1 and Drice, and the degeneration of nurse cell nuclei or entire egg chambers. In the *pita/dcp-1* double mutants, the absence of *dcp-1* prevented the degeneration of egg chambers, and abnormal egg chambers persisted until the end of oogenesis. These abnormal egg chambers may be developmentally delayed and unable to progress to the normal late events of actin bundle formation and dumping. We suggest that the apparent defect in late nurse cell death is due to the survival of these developmentally delayed egg chambers. Consistent with this, *pita* mutants show persistent polyteny of nurse cell nuclei and late-stage *pita* egg chambers show high levels of CM1 (Figure 5F), normally seen only during cell death in mid-oogenesis (PETERSON *et al.* 2003).

Cell death during mid-oogenesis may be regulated by ecdysone signaling (Buszczak *et al.* 1999; Carney and Bender 2000). In the mosquito, levels of ecdysone increase following a blood meal, which leads to vitellogenesis and increased egg production (reviewed in Raikhel *et al.* 2002). Drosophila mutants lacking genes within the ecdysone response hierarchy show premature egg chamber degeneration (Buszczak *et al.* 1999; Carney and Bender 2000) similar to *pita* mutants. Interestingly, the larval lethality seen in *pita* mutants can be partially rescued by exogenous ecdysone (B. Laundrie and K. McCall, unpublished results), suggesting that *pita* may regulate or interact with components within the ecdysone signaling hierarchy. Several cell death genes are known to be induced during ecdysone-regulated salivary gland cell death (Gorski *et al.* 2003; Lee *et al.* 2003); however, it is unknown if the same mechanism acts in the ovary.

Flies homozygous for *dcp-1*^{Prev1} appeared normal and were fertile, suggesting that other effector caspases function redundantly with *dcp-1* during developmental cell death. However, the loss of *dcp-1* prevented germline cell death from occurring during mid-oogenesis in response to nutrient deprivation, suggesting that other caspases are not always capable of substituting for *dcp-1*. Closer examination of *dcp-1*^{Prev1} flies may reveal other types of cell death that are also strictly dependent on *dcp-1*. This situation is similar to that occurring in the mouse, where caspase-3 is essential for some types of cell death, but other caspases may substitute in different types of cell death (Zheng *et al.* 2000; Ranger *et al.* 2001).

The loss of *dcp-1* led to defective cell death in mid-oogenesis, with a corresponding decrease in activity and mislocalization of another effector caspase, Drice, seen with the CM1 antibody. As Dcp-1 has previously been shown to process Drice *in vitro* (Song *et al.* 2000), this direct mechanism may be critical during cell death in mid-oogenesis. Furthermore, proper localization of Drice may depend on cleavage of nuclear targets, such as nuclear lamins, by Dcp-1. This suggestion is supported by our observation that nuclear lamin overexpression also causes mislocalization of CM1 staining to the nucleus (M. Barkett and K. McCall, unpublished results). Similar to the loss of *dcp-1*, overexpression of the caspase inhibitor DIAP1 blocks germline cell death in mid-, but not late, oogenesis (Peterson *et al.* 2003). These findings suggest that mid-oogenesis cell death utilizes a Dcp-1-Drice pathway that can be inhibited by DIAP1, resembling the pathway utilized during cell death in the eye (Yu *et al.* 2002). Surprisingly, Dcp-1 and DIAP1 do not affect late nurse cell death, suggesting that a novel mechanism acts in late oogenesis.

Insight into the late oogenesis cell death pathway may come from mutations in subunits of the transcription factor E2F. Mutations in either the *dE2F1* or *DP* subunits of E2F inhibit late nurse cell death, showing many of the same phenotypes seen in the *dcp-1/pita* double mutants (Myster *et al.* 2000; Royzman *et al.* 2002; K. Cullen, J. Peterson and K. McCall, unpublished observations). The *dE2F1/DP* phenotypes include dumpless egg chambers, thick follicle cell layer, and abnormal nurse cell nuclear morphology, but not early nurse cell death. Pita and E2F may function together to activate genes required for proper egg chamber development, but E2F may have an additional function in regulating germline cell death, thereby resembling the *pita/dcp-1* double mutant. Alternatively, both *pita* and *dcp-1* may be transcriptional targets of E2F, with coregulation resulting from the nested gene arrangement.

Nested gene arrangements are relatively common in the Drosophila genome. We have identified 898 nested protein-coding genes in Drosophila, similar to the number (879) reported by Misra *et al.* (2003). We found that at least 37 of the 898 gene pairs have a reported *P*-element insertion within 1 kb of the nested gene. Therefore, the possibility exists that these *P* elements disrupt the outlying and/or nested genes. Indeed, the *P*-element-induced *gutfeeling* phenotype originally attributed to the *OAZ* gene (Salzberg *et al.* 1996) was recently found to be caused by the disruption of the gene *SmD3*, which is nested within an intron of *OAZ* (Schenkel *et al.* 2002). Here we have found that single *P* elements simultaneously disrupt two genes that both affect oogenesis. As large efforts are underway to disrupt most Drosophila genes with *P* elements (Spradling *et al.* 1999), caution must be taken in assigning insertional phenotypes to individual genes, particularly in the case of nested or otherwise closely associated genes.

We thank Bruce Reed, Terry Orr-Weaver, Welcome Bender, Pernille Rorth, the Bloomington Stock Center, the Berkeley *Drosophila* Genome Project, Bruce Hay, and Idun Pharmaceuticals for fly strains and reagents; Artem Buynevich, Aeona Wasserman, Anna Terajewiecz, Sarah Carlson, and Ian Watt for excellent technical assistance; and Chris Li, Susan Tsunoda, Simon Kasif, Lynn Cooley, and members of the lab for helpful suggestions. This work was supported by the Clare Boothe Luce Program of the Henry Luce Foundation, research project grant no. 00-074-01-DDC from the American Cancer Society, National Institutes of Health grant R01 GM60574, and a Basil O'Connor Starter Scholar Award from the March of Dimes (K.M.). S. R. Thompson's work was supported in part by a National Science Foundation KDI grant no. 9980088.

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Communicating editor: T. Schüpbach