Apoptosis in late stage *Drosophila* nurse cells does not require genes within the *H99* deficiency

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SUMMARY

We have determined that nurse cells are cleared from the *Drosophila* egg chamber by apoptosis. DNA fragmentation begins in nurse cells at stage 12, following the completion of cytoplasm transfer from the nurse cells to the oocyte. During stage 13, nurse cells increasingly contain highly fragmented DNA and disappear from the egg chamber concomitantly with the formation of apoptotic vesicles containing highly fragmented nuclear material. In dumpless mutant egg chambers that fail to complete cytoplasm transport from the nurse cells, DNA fragmentation is markedly delayed and begins during stage 13, when the majority of cytoplasm is lost from the nurse cells. These data suggest the presence of cytoplasmic factors in nurse cells that inhibit the initiation of DNA fragmentation. In addition, we have examined the ovarian expression patterns of regulatory genes implicated in *Drosophila* apoptosis. The positive regulators, *reaper (rpr)*, *head involution defective (hid)* and *grim*, as well as the negative regulators, *DIAP1* and *DIAP2*, are transcribed during oogenesis. However, germline clones homozygous for the deficiency *Df(3)H99*, which deletes *rpr*, *hid* and *grim*, undergo oogenesis in a manner morphologically indistinguishable from wild type, indicating that genes within this region are not necessary for apoptosis in nurse cells.

Key words: *Drosophila*, Apoptosis, *H99*, Nurse cell, Oocyte, DNA fragmentation, *reaper* (*rpr*), *head involution defective* (*hid*), *grim*

INTRODUCTION

The *Drosophila* egg chamber consists of a cluster of 16 interconnected germline cells surrounded by a monolayer of somatic follicular epithelium (see Spradling, 1993). One cell in the germline cluster develops into the oocyte while the other fifteen become nurse cells that transcriptionally and translationally support the oocyte in its development. Beginning at stage 10B, near the end of oogenesis, several events occur that suggest the initiation of an apoptotic pathway. Nurse cells undergo actin cytoskeletal rearrangements (Gutzeit, 1986; Cooley et al., 1992), appear to round up (Okada and Waddington, 1959) and their nuclear membranes become permeabilized (Okada and Waddington, 1959; Giorgi and Deri 1976; Cooley et al., 1992). Morphological changes like these have been linked to cell death in degenerating egg chambers (Waddington and Okada 1960, Giorgi and Deri 1976) and to apoptosis in general (see Arends and Wyllie, 1991 for review). Following these events, the majority of the nurse cell cytoplasm is transported into the oocyte within approximately 30 minutes (reviewed in Mahajan-Miklos and Cooley, 1994a), the nurse cells regress and are lost from the egg chamber in a manner that resembles the clearing of apoptotic cell corpses.

Apoptosis (Kerr et al., 1972) refers to an evolutionarily conserved method of cell death that is genetically regulated and accompanied by distinctive, stereotypic changes in cell shape and DNA conformation (reviewed in Ellis et al., 1991; Arends and Wyllie, 1991). Apoptotic cells lose contact with neighboring cells and the extracellular matrix (Kerr et al., 1972; Abrams et al., 1993), the cytoplasm and nucleus condense (Kerr et al., 1972; Wyllie, 1980; Abrams et al., 1993), the nuclear membrane becomes permeable (Kerr et al., 1972; Wyllie, 1980), and DNA localizes subjacent to the nuclear membrane where it amasses into large, highly condensed clumps (Kerr et al., 1972; Wyllie, 1980). During this condensation period, the DNA of dying cells is fragmented initially into 300 kb and 50 kb pieces (Oberhammer et al., 1993), and then into 180-200 bp nucleosomal multimers (Wyllie, 1980). Finally, convolution of the nuclear envelope (Jacobson et al., 1994) and plasma membrane generates protrusions or blebs of cytoplasmic and nuclear material that are pinched off and phagocytosed by neighboring cells or by phagocytes (Kerr et al., 1972; Wyllie, 1980). In this way, cells that are redundant, no longer necessary, or deleterious to the well being of the organism are selectively deleted in a manner that maintains the integrity of cell membranes (Jacobson et al., 1997). This ensures that deletion proceeds without leaking potentially noxious cellular contents into the surrounding tissue. In contrast, necrosis, another means by which cells die, generally arises in response to an insurmountable insult to cellular homeostasis, is typified by cellular swelling and rupture of the plasma membrane, and results in inflammation of the surrounding tissue.

Many genes that function during apoptosis have been identified in organisms ranging from *C. elegans* to mammals (Hengartner, 1995; McCall and Steller, 1997). In *Drosophila*, three positive

regulators of apoptosis have been identified. These genes, *reaper (rpr)* (White et al., 1994), *head involution defective (hid)* (Grether et al., 1995) and *grim* (Chen et al., 1996), all lie within the region uncovered by the relatively small deletion *Df (3L)H99 (H99)* at 75C1,2. In embryos that are deficient for this region, apoptosis is blocked, but can be restored by exposure to high doses of ionizing radiation (White et al., 1994). The products of these three genes contain a small region of homology to one another in the first 14 amino-terminal residues (Grether et al., 1995, Chen et al., 1996) and, although the entire Rpr protein has some homology to the death domain motifs found in several mammalian proteins involved in apoptotic pathways (Golstein et al., 1995), such as Fas receptor (Fas-R) (Itoh and Nagata, 1993) and Tumor Necrosis Factor Receptor (TNF-R) (Tartaglia et al., 1993), mutations in regions other than the first few N-terminal amino acids have little effect on cell death (Vucic et al., 1997). Grim and Hid show no other homology to known proteins (Grether et al., 1995, Chen et al., 1996). Binding of Rpr with Grim, Hid or other proteins has not been documented; however, Rpr and Hid have been shown to act cooperatively in the death of midline glial cells in the developing embryo (Zhou et al., 1997). The means by which Rpr, Grim and Hid act to promote apoptosis in *Drosophila* is not known at present.

In addition to these three positive effectors of apoptosis in *Drosophila*, two negative regulators, *DIAP1* and *DIAP2*, have also been found (Hay et al, 1995). When overexpressed in the eye under the control of the *glass* promoter, these genes inhibit apoptosis induced by co-expression of *rpr* or *hid* in the developing eye. *DIAP1* and *DIAP2* code for proteins that contain Baculovirus Inhibitor of apoptosis Repeat (BIR) domains as well as a Zn^{++} RING finger motif. The BIR repeats of c-IAP1 and c-IAP2 in mammals have been shown to mediate protein-protein interactions (Rothe et al., 1995), and RING fingers are generally involved in protein-DNA binding and possible protein-protein interactions (see Schwabe and Klug, 1994 for review). Although it has been shown that only the BIR domains and a small linker region of *DIAP1* are necessary for its activity, the means by which *DIAP1* and *DIAP2* accomplish inhibition is not clear (Hay et al., 1995).

We have determined that nurse cells are cleared from the egg chamber by apoptosis. DNA fragmentation indicative of apoptosis begins in nurse cells at stage 12 after the completion of rapid cytoplasmic transport and increases throughout stage 13. During stage 13, apoptotic vesicles containing nuclear material can be seen in the nurse cell cluster. Examination of mutant egg chambers deficient in rapid transport revealed that DNA fragmentation is correlated with loss of cytoplasm, possibly containing inhibitory factors. By in situ analysis, we show that *rpr, grim, hid, DIAP1* and *DIAP2* mRNAs are expressed in the *Drosophila* ovary. However, we were surprised to learn that when we generated germline clones of the *H99* deletion, genes within this interval were not necessary for nurse cells to undergo apoptosis. This leads us to suspect the presence of a positive effector of apoptosis in the *Drosophila* ovary that has not yet been characterized.

MATERIALS AND METHODS

Drosophila stocks

Canton S was used as the wild-type control. The *Df(3L)H99/TM3* stock was a gift from Hermann Steller. Dumpless mutant stocks were

$chic^{1320}$ (Cooley et al., 1992), $cher^1$ (Robinson et al., 1997), $kelch^{DE1}$ (Xue and Cooley, 1993), $quail^{QE24}$ (Schüpbach and Wieschaus, 1991) and sn^{28} (Cant et al., 1994). All stocks were maintained according to standard procedures. Females were fed wet yeast paste prior to ovary dissection to increase egg production.

DNA extraction and electrophoresis

DNA from 180 *Canton S* ovaries was extracted using standard methods. Ovaries were dounced in an Eppendorf tube containing 200 μl ice-cold grinding buffer (10 mM Tris pH 7.8, 60 mM NaCl, 10 mM EDTA). Immediately following douncing, 200 μl of lysis buffer (1.25% SDS, 0.3 M Tris pH 9, 0.1 M EDTA, 5% sucrose, 10% DEPc) was added and the sample was gently mixed by inversion and placed at 65°C for 40 minutes. Cellular debris was pelleted by adding 60 μl 8 M KOAc, incubating the sample on ice for 30 minutes and centrifuging at 15,000 *g* for 6 minutes. The supernatant was phenol-chloroform extracted twice and chloroform extracted once. DNA was precipitated with 2.5 volumes 100% ethanol, washed with 70% ethanol, dried on the bench top and resuspended in TE (10 mM Tris pH 7.6, 1 mM EDTA). DNA was electrophoresed at 80 V for 5 hours on a 1.8% agarose gel containing ethidium bromide and visualized with a UV transilluminator.

Acridine orange staining

Acridine orange staining was conducted according to Abrams et al. (1993) with minor modifications. Ovaries were dissected into individual egg chambers in EBR (130 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 10 mM Hepes pH 6.9) then placed in 50% acridine orange solution (10 μg/ml) in heptanes for 3 minutes in the dark. Samples were immediately placed onto glass slides after which the excess dye was aspirated away. Egg chambers were then covered with halocarbon oil and a coverslip. Elapsed time from dissection of the ovaries to the end of viewing was restricted to 20 minutes. Samples were examined with a Zeiss Axiophot fluorescence microscope using a UV-G excitation filter.

TUNEL labeling of fragmented DNA

TUNEL was conducted according to Gavrieli et al. (1992) with some modifications. Ovaries were fixed in 400 μl solution 1 (2 mM Mg_2SO_4, 0.1 M Hepes, pH 6.9, 1 mM EGTA), 400 μl heptanes and 100 μl 36% formaldehyde (undiluted) for 25 minutes, rocking to mix phases. After fixing, samples were washed with PBTx (1× PBS 0.3% Triton X-100) then incubated for 3 hours at 37°C in TdT reaction buffer (0.1 M KCacodylate [pH 7.2]; 2 mM $CoCl_2$; 0.2 mM DTT [Boehringer-Mannheim]), 3 nM Biotin-16-dUTP (Boehringer-Mannheim) 1 nM dNTPs and TdT(45 U) in the case of experimental samples or an equal aliquot of ddH_2O in place of TdT in the negative control samples.

Following this reaction, ovaries were rinsed briefly, washed three times over the course of 1 hour in PBTx and blocked for an hour in PBTxB (1× PBS, 0.1% Triton X-100, 0.3% bovine serum albumin [BSA]). Fluorescein-conjugated streptavidin was added at a concentration of 1 μg/100 μl and the samples were incubated at room temperature for 1.5 hours. During this incubation and all subsequent procedures, care was taken to limit the exposure of the samples to light. The samples were washed 4× over 1.5 hours, equilibrated with 50% glycerol in PBS for approximately 30 minutes and mounted in 50% glycerol in PBS. Egg chambers were viewed by confocal microscopy (Biorad MRC-600).

In situ hybridization

rpr, hid, grim, DIAP1 and *DIAP2* cDNAs were from: *rpr,* H. Steller; *hid* and *grim,* J. Abrams; *DIAP1* and *DIAP2,* B. Hay. Riboprobes were generated against these cDNAs and in situ hybridizations were performed according to O'Neill and Bier (1994) with the following changes. Ovaries were dissected in EBR and fixed in 400 μl solution 1 (2 mM MgSO₄, 0.1 M Hepes, pH 6.9, 1 mM EGTA) 400 μl heptanes and 100 μl 36% formaldehyde (undiluted) for 25 minutes. Ovaries were dehydrated, cleared and refixed as stated in O'Neill and Bier (1994). Following proteinase K treatment and subsequent second

refixation, ovaries were washed in PBTw (1× PBS, 0.1% Tween in DEPC-treated ddH₂O) and dissected into individual ovarioles. Prehybridization, hybridization and initial washes in hybridization buffer were all done at 58°C. Alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody (gift of Dr Carl Hashimoto) was preadsorbed to ovaries overnight and used at 1 : 2000 dilution in 1× PBTxB (0.1% Triton X-100, 0.3% BSA). Following completion of the AP reaction and subsequent 100% ethanol washes, egg chambers were serially rehydrated and washed 3× in PBTw. Egg chambers were equilibrated and mounted in 50% glycerol in PBS. Controls for the in situ protocol were as follows: egg chambers were probed with an antisense *bicoid* riboprobe as a positive control for the in situ protocol. *rpr*, *hid* and *grim* antisense riboprobes were first hybridized to embryos to ascertain their quality. Sense strand riboprobes were generated for the genes used in these experiments as negative controls. Additional negative controls for all in situs were samples that lacked riboprobe but were treated identically to the experimental samples otherwise.

Germline clones

Germline clones deficient for the *H99* region were made by mitotic recombination according to Perrimon et al. (1989). As a negative control, germline clones were also made of a chromosome wild type for the *H99* region called *l(3)16*. *H99/TM3* and *l(3)16/TM3* virgin females were mated to *P[ovo^{D1}]^{3L} FRT^{3L}/TM3* males (Chou and Perrimon, 1996). First instar larvae were irradiated at 1000 rads using an X-ray source or a γ-ray source, which yielded comparable results. 2-3 days after mating, the ovaries of non-*TM3* female progeny were dissected in 1× EBR and examined for the presence egg chambers more mature than *ovo^D*-arrested egg chambers. To confirm the germline genotype of mature egg chambers, expression from the *H99* region was analyzed by in situ hybridization as previously described, using a *hid* antisense riboprobe. Following rehydration, samples were tested for DNA fragmentation by TUNEL labeling.
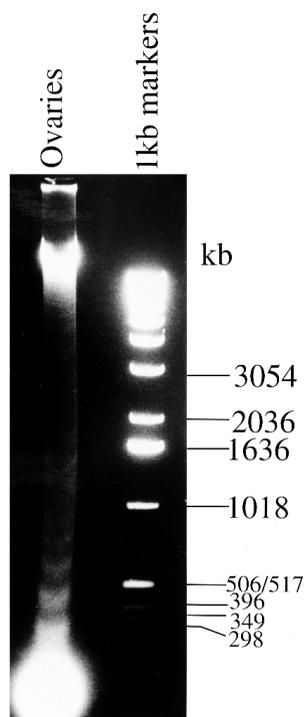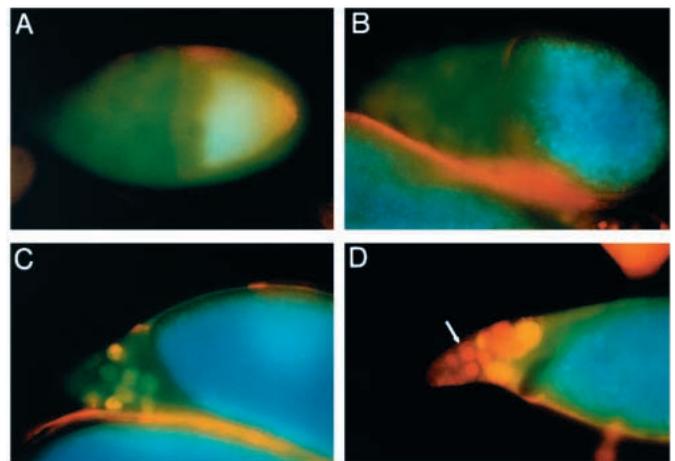
RESULTS

Nurse cell DNA is fragmented after rapid cytoplasmic transfer

To determine whether apoptosis occurs in the ovary, we first electrophoresed DNA extracted from ovaries on an agarose gel. This yielded a pattern of multimers approximately 180 bp in length, indicative of the nucleosomal DNA fragmentation seen during apoptosis (Fig. 1). We then identified individual apoptotic cells by two separate and complementary means of labeling fragmented DNA: acridine orange staining (Kasten, 1967; Abrams et al., 1993) and TUNEL labeling (Gavrieli et al., 1992).

Acridine orange is a basic intercalating dye that preferentially labels apoptotic cells (Abrams et al., 1993) and can be used to determine the relative integrity of chromatin (Kasten, 1967). As fragmentation occurs, DNA becomes more accessible to dye stacking and the concentration of bound dye particles increases. This increase in dye concentration is seen as a change in the wavelength of light emitted, which is especially evident when samples are viewed with a UV-G excitation filter used to view samples stained with DAPI. In these experiments, green nuclear fluorescence indicates low dye concentration and supercoiled DNA, yellow and yellow-orange represents increasing DNA fragmentation and red is the maximum concentration of dye and highest degree of DNA fragmentation (Kasten, 1967).

Using acridine orange staining, stage 10 (Fig. 2A) and 11 (Fig. 2B) nurse cell nuclei appeared green, indicative of intact DNA. DNA fragmentation was detectable beginning asynchronously at stage 12 with few nurse cells containing bright yellow nuclei (Fig. 2C). Throughout stage 13 (Fig. 2D) DNA fragmentation increased and nurse cell nuclei decreased in size and were asynchronously lost from the egg chamber. During this time,

Fig. 1. Nucleosomal fragmentation of DNA occurs in *Drosophila* ovary. DNA extracted from ovaries resolves into a ladder-like pattern of approximately 180 bp multimers during electrophoresis. This pattern indicates that DNA in the ovary is being cleaved at nucleosomal intervals and is a common indicator of apoptosis.

Fig. 2. Acridine orange staining identifies the location of DNA fragmentation. During stages 10 (A) and 11 (B), DNA in nurse cell nuclei fluoresce green after acridine orange staining, indicating intact DNA. At stage 12 (C), some nurse cell nuclei begin to stain yellow, indicating that fragmentation has begun. Cleavage of DNA appears to proceed asynchronously in nurse cells throughout stages 12 and 13 (D) until all nuclei show signs of fragmentation. During stage 13, nurse cell nuclei have begun to stain red, indicating that the DNA in those cells is highly fragmented. At this time also, nurse cell nuclei diminish in size and there are vesicles containing bright red-staining nucleic acid in the nurse cell cluster (arrow).

small blebs of red-staining nuclear material were also seen near shrinking nuclei within the cluster (Fig. 2D, arrow).

TUNEL labeling, which fluorescently tags 3′-ends of fragmented DNA, yielded results similar to that seen with acridine orange staining. Again, no evidence of DNA degradation was present in stage 10 (Fig. 3B). DNA degradation was first detected during stage 12 in two or three of the 15 nurse cells, and increased both in number of nurse cell nuclei affected and in intensity of staining during stage 13 (Fig. 3C). Finally, the number of nurse cells diminished and few brightly staining nurse cell nuclei were seen (Fig. 3D).

DNA fragmentation in dumpless mutant ovaries

We next determined whether apoptosis occurs in nurse cells that are unable to properly transfer cytoplasm to the oocyte. We examined two types of 'dumpless' mutants: those with aberrant ring canal morphology causing impaired cytoplasm transport throughout oogenesis and those with nurse cell actin cytoskeleton defects that affect only rapid cytoplasm transport during stage 11. Other aspects of development in these mutants appear to be normal. For example, egg shell deposition around the oocyte, which normally occurs after dumping, proceeds in dumpless mutants even though only very small oocytes are formed. *cheerio* and *kelch* mutants have aberrant ring canal morphology that does not permit cytoplasm to pass easily from the nurse cells to the oocyte (Xue and Cooley, 1993; Robinson et al., 1994, 1997). The *chickadee, singed* and *quail* gene products are necessary for the proper formation of cytoplasmic actin filament bundles that form in nurse cells at stage 10B, just prior to the onset of rapid cytoplasmic transport (Cooley et al., 1992; Cant et al., 1994; Mahajan-Miklos and Cooley, 1994b). These bundles tether nurse cell nuclei in the approximate center of the cell, allowing free flow of cytoplasm through the ring canals (see Mahajan-Miklos and Cooley, 1994a for review). *chickadee* encodes the *Drosophila* homologue of profilin (Cooley et al., 1992), a protein that promotes actin filament formation and sequesters actin monomers (Pollard and Cooper, 1984). The products of *singed* and *quail* have homology to fascin and villin, respectively, and are actin bundling proteins (Cant et al., 1994; Mahajan-Miklos and Cooley, 1994b).

We examined egg chambers from these mutants for DNA fragmentation by TUNEL labeling and data for *kelch* and *chickadee*, representing the two types of dumpless mutants, are shown in Fig. 4. Staging was determined by noting the presence or absence of dorsal appendages and nurse cells in the anterior of the egg chamber. Dumpless egg chambers in which the dorsal appendages had not yet formed were considered stage 12, whereas mutant egg chambers that contained both the nurse cell cluster and dorsal appendages were designated stage 13 (after King, 1970). DNA fragmentation was not seen during stage 12. Instead, degradation began in stage 13 after the dorsal appendages had grown into the nurse cell cluster, but only in one or two cells of the 15 nurse cells present. The cells that initially contained fragmented DNA were generally close to the overlying follicle cell layer rather than

in the interior of the cluster (Fig. 4B,E). Throughout late stage 13, nurse cells in dumpless egg chambers gradually lost both nuclear and cytoplasmic volume (compare Fig. 4A through C, and D through F). At a point when the majority of cell volume was lost, 15 brightly staining nurse cell nuclear remnants were still seen in the egg chamber, along with bright vesicles of nuclear material (Fig. 4C,F).

Positive and negative regulators of apoptosis are expressed in *Drosophila* ovary

To determine the ovarian expression patterns of genes involved in the positive regulation of apoptosis, we used digoxigenin-labeled antisense riboprobes to *rpr, hid* and *grim* in in situ hybridization experiments. *rpr* (Fig. 5A) and *hid* (Fig. 5C) were expressed in nurse cells beginning at stage 9 and continuing throughout stage 13. The *grim* transcript was not expressed as strongly as *rpr* or *hid* but it mirrored the pattern for *rpr* and *hid* (data not shown). These expression patterns suggested that regulatory genes that positively affect apoptosis in *Drosophila* embryos may also be active in late stage egg chambers. Unlike the temporal expression pattern seen in embryos, which precedes the onset of DNA fragmentation by 1-2 hours (White et al., 1994), expression of *rpr* in the ovary precedes detectable DNA fragmentation by approximately 5.4 hours (staging according to King, 1970). When the dumpless mutants *chickadee*, *singed*, *kelch* and *quail* were examined for expression of these transcripts, it was noted that expression began normally at stage 9 and continued until the end of oogenesis (data not shown).

We next examined the expression patterns of inhibitors of
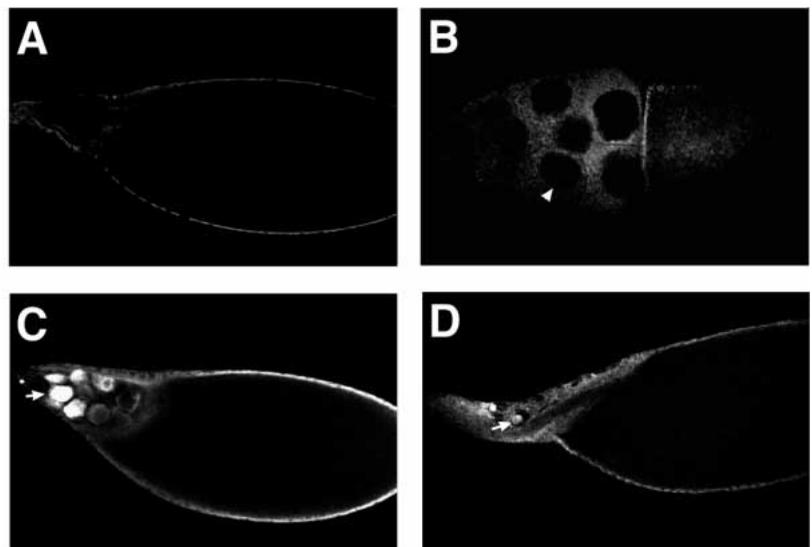
Fig. 3. TUNEL labeling confirms that DNA in nurse cells is fragmented during stages 12 and 13. The 3′ ends of fragmented DNA were labeled in a terminal transferase-mediated reaction and examined by confocal microscopy. Negative control samples were treated identically as experimental, with the exception that they did not receive terminal transferase enzyme. (A) A negative control egg chamber at stage 13. (B) No staining of nurse cell nuclei was seen at stage 10. An arrowhead points to a nurse cell nucleus that is unlabeled. (C) Early in stage 13, not all nurse cell nuclei are stained with the same intensity, indicative of the asynchronous nature of DNA fragmentation in these cells. (D) As stage 13 progresses, nurse cells disappear from the egg chamber and those that remain appear vastly shrunken in comparison with earlier stages. Arrows point to brightly stained nurse cell nuclei that contain fragmented DNA.
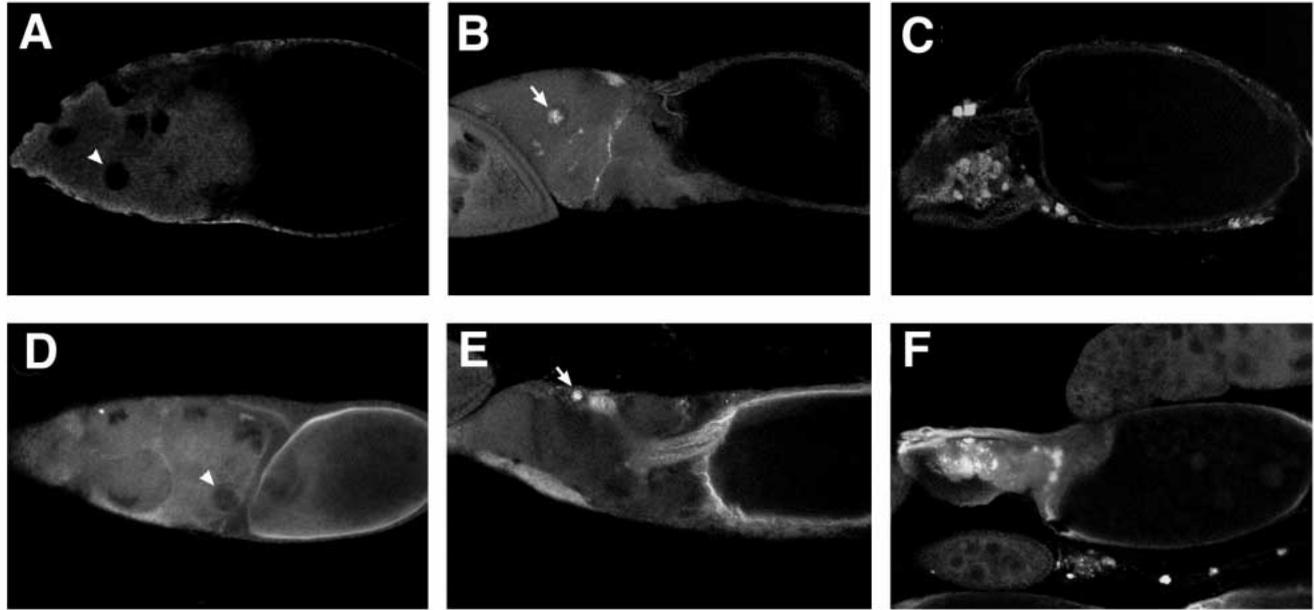
Fig. 4. TUNEL labeling of dumpless mutants indicates that DNA fragmentation occurs in nurse cells, but is delayed with respect to wild type. (A-C) *chic^1320*; (D-F) *kelch^DE1.* (A,D) Early in stage 13, the nurse cells in dumpless egg chambers still contain the majority of their cytoplasm and their nuclei are not labeled by TUNEL. Arrowheads point to unlabeled nuclei. (B,E) Later in stage 13, some cytoplasm has been lost and one or two of the fifteen nurse cell nuclei present contain fragmented DNA (arrows). (C,F) Finally, late in stage 13, the nurse cell cluster is greatly reduced in size and contains remnants of nurse cells in which the DNA is greatly fragmented.

apoptosis, *DIAP1* and *DIAP2* by in situ hybridization. *DIAP1* was expressed in nurse cells beginning at region 2B in the germarium and remained at this level until stage 7 (Fig. 5B). During stage 7 and 8, *DIAP1* was down-regulated. Expression resumed at pre-stage 7 levels during stage 9 and continued at this level until the completion of rapid transport. *DIAP2* was expressed at a higher level in nurse cells than *DIAP1*, but its temporal expression pattern was similar (Fig. 5D). Following rapid transport, the levels of both *DIAP1* and *DIAP2* in the nurse cells greatly diminished (Fig. 5E and F). The majority of staining seen at this time in the egg chamber appeared in the overlying follicle cells.

When expression of these transcripts was examined in the dumpless mutants *chickadee* and *kelch*, early expression from 2B in the germarium to stage 7 progressed normally, and later expression from stage 9 through 10 was also comparable to wild type. Interestingly, however, these transcripts remained elevated until late stage 13 and disappeared from individual nurse cells asynchronously (data not shown). This timing correlated well with the onset of nurse cell DNA fragmentation in these mutants.

### Germline clones of the *H99* deficiency undergo apoptosis

Because homozygous loss of genes in the *H99* deficiency results in an embryonic lethal phenotype (White et al., 1994), we created homozygous *H99* germline clones by X-ray-induced mitotic recombination to examine the function of *rpr*, *grim* and *hid* during oogenesis. In this experiment, *H99/TM3* females were mated to *ovo^D/TM3* males and the progeny were irradiated as first instar larva. Of the adult non-*TM3* female progeny dissected, 3-6% contained clonal egg chambers that were first identified as morphologically distinct from the

agametic *ovo^D* phenotype, and then by lack of *hid* expression in in situ hybridization experiments. Contrary to expectations, the overall morphology of *H99/H99* germline clones was indistinguishable from that of wild type (Fig. 6A). TUNEL labeling showed that DNA fragmentation still occurred in stage 12 and 13 nurse cells (Fig. 6C,D). These findings indicated that, unlike apoptosis in embryos (White et al., 1994), genes within the *H99* deficiency are not required for apoptosis to occur during oogenesis. This suggests the presence of at least one other as yet uncharacterized gene that regulates apoptosis in the *Drosophila* female germline.

DISCUSSION

Morphological phenomena in late stage egg chambers are consistent with apoptosis

Late stage nurse cells display a full range of morphological changes common to apoptotic cells. Most prominently, DNA fragmentation can be detected in nurse cells following the completion of rapid cytoplasm transport at stage 12. Throughout stage 13, small vesicles containing nuclear material are found in the egg chamber and nurse cells gradually diminish in size and disappear. Vesicularization of nurse cells was also documented by Giorgi and Deri (1976) in an ultrastructural study of degenerating egg chambers. This report also showed that blebs of nurse cell material are phagocytosed by overlying follicle cells. It is entirely likely then that nurse cells are cleared from the egg chamber during stage 13 by follicle cell heterophagy of apoptotic vesicles. Interestingly, manifestations of possible apoptotic behavior are evident prior to the onset of rapid cytoplasm transport. Beginning at stage
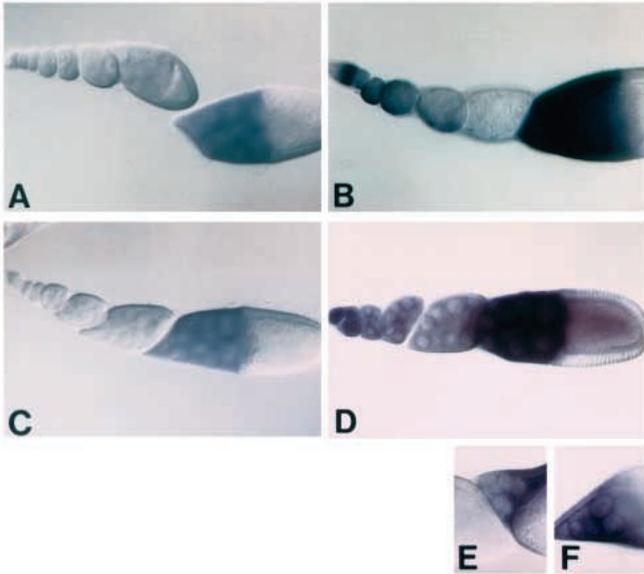
Fig. 5. Both positive and negative regulators of apoptosis are expressed in nurse cells. In situ hybridization revealed that *rpr* (A) and *hid* (C) are both consistently first detected above background levels late in stage 9. This expression increases during stage 10 and continues throughout oogenesis. *DIAP1* (B,E) and *DIAP2* (D,F) are expressed beginning at stage 2B in the germarium. This expression continues until stage 7 and resumes at stage 9 (B,D). At stage 12 (E,F), when rapid cytoplasmic transport is complete, less transcript is seen in the nurse cells.

10B, the actin cytoskeleton is reorganized (Gutzeit, 1986; Cooley et al., 1992), the nuclear envelope is permeabilized (Okada and Waddington, 1959; Giorgi and Deri 1976; Cooley et al., 1992) and the cells round up, suggesting that an alteration of adhesive properties has also occurred (Okada and Waddington, 1959). Although these phenomena are also seen during such events as delamination and cell division, neither of these processes occurs in nurse cells at this time.

The presence of what could be apoptotic phenomena prior to the onset of rapid cytoplasmic transport offers the possibility that these pathways may be interconnected, and that signals initiating apoptosis in nurse cells may in fact trigger nurse cell contraction as part of a cascade of apoptotic events. One way to test this idea is to look for evidence that the downstream effectors of apoptosis have a role in cytoplasm transport. Mutations in the *Drosophila* ICE-like caspase *dcp-1* have been reported (Song et al., 1997). This caspase is transcribed during oogenesis (Song et al., 1997; K. McCall and H. Steller, see Note added in proof) and is required for proper larval development (Song et al., 1997). Germline clones of *dcp-1* fail to undergo rapid cytoplasm transport (K. McCall and H. Steller, personal communication). This result is consistent with a model in which apoptosis initiates the onset of rapid cytoplasmic transfer during *Drosophila* oogenesis.

rpr, hid and *grim* are not necessary for apoptosis during *Drosophila* oogenesis

To investigate whether known molecules involved in apoptosis are required to initiate nurse cell cytoplasm, we studied the expression of *rpr, hid* and *grim* in ovaries. While all three genes are transcribed in nurse cells, deletion of the genes by making
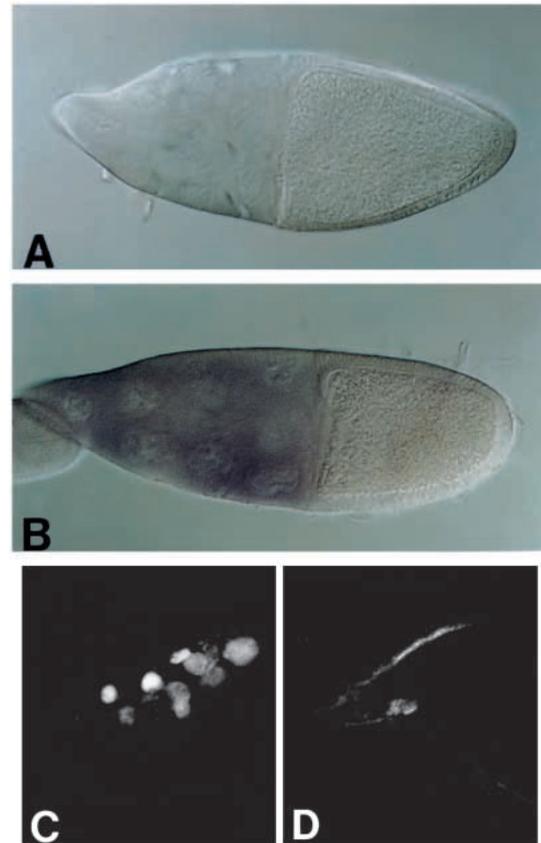
Fig. 6. Germline clones of *H99* undergo DNA fragmentation. Egg chambers from germline clones of *H99* (A) and *l(3)16* (B) were subjected to in situ analysis using a riboprobe generated against the *hid* cDNA. *l(3)16* is wild type for the *H99* interval. Once clones of *H99* were identified, TUNEL labeling was carried out to check for DNA fragmentation (C,D). While *H99* clones failed to express *hid*, they nevertheless underwent DNA fragmentation. (C) An early stage 13 egg chamber with many labeled nuclei, while (D) shows a later stage 13 egg chamber in which fewer nurse cells remain. These data indicate that apoptosis in nurse cells does not require genes within the *H99* deficiency.

H99 germline clones has no apparent effect on oogenesis, or on the death of nurse cells by apoptosis. This indicates that these genes are not required for apoptosis in the female germline. A likely possibility is that these mRNAs are not translated in the developing egg chamber, but are required for maternal loading. During oogenesis, many mRNAs are transcribed for transport into the oocyte, where they are translated for use at later stages in development (Roth et al., 1989; Wharton and Struhl, 1989; Wang and Lehmann, 1991). This may also be true of *rpr, hid* and *grim.*

Additionally, apoptosis can proceed in embryos lacking *rpr, hid* and *grim* following exposure to high doses of ionizing radiation (White et al., 1994). This indicates that genes within the *H99* deficiency are not needed to carry out the mechanics of apoptosis, but fulfill a regulatory, perhaps signal transducing role (White et al., 1994). There is some evidence for tissue-specific requirements for these individual genes. *rpr, hid* and *grim* transgenes can each restore apoptosis to most *H99⁻* embryonic tissues; however, *rpr* and *grim* transgenes cannot compensate for

loss of *hid* in restoring cell death to the head region (Chen et al., 1996). Additionally, apoptosis that occurs in cells of the central nervous system during embryonic stage 16 does not require *hid* expression (Chen et al., 1996), as these cells express *rpr* and *grim*, but do not express *hid*. In keeping with these findings, there may be positive regulatory genes required for apoptosis in the female germline that are not in the *H99* region and that do not require the genes within the *H99* region to function.

DNA fragmentation begins in nurse cells following cytoplasm depletion

Our work with dumpless mutants shows that DNA fragmentation only proceeds after the depletion of cytoplasm from the nurse cells. These data suggest the presence of inhibitory factors in the cytoplasm that are depleted by rapid cytoplasmic transfer during wild-type oogenesis. It is also possible that a more global delay in nurse cell development may be a factor in the delay of DNA degradation in dumpless mutant nurse cells. In dumpless mutants, the mechanism for removal of cytoplasm is unclear, but it could involve follicle cell heterophagy or leakage from the egg chamber.

We propose a model where the cytological changes that occur in nurse cells prior to dumping are examples of apoptotic phenomena. While nuclear permeabilization occurs at stage 10B in both wild-type and the dumpless mutants studied (Cooley et al., 1992; Xue and Cooley, 1993; K. F., unpublished), DNA fragmentation is delayed until the completion of rapid cytoplasm transport in wild-type egg chambers and until cytoplasmic depletion in the mutants. Since we hypothesize that both wild-type and mutant nurse cells initiate the apoptotic program at similar times, this suggests that DNA fragmentation might be separated from the onset of apoptosis by cytoplasmic factors found in nurse cells. *DIAP1* and *DIAP2* are likely candidates for these inhibitory factors. The activity of *DIAP1* in oogenesis has previously been shown by clonal analysis (Hay et al., 1995). Germline clones deficient in *DIAP1* are indistinguishable from *ovo^D* suggesting that this gene is necessary to prevent the death of stem cells or young egg chambers in the early stages of oogenesis. Our in situ results point to a role for *DIAP1* and *DIAP2* in later stages of oogenesis also. These two proteins may prevent DNA fragmentation until their concentration in the nurse cell cluster is diminished in both wild-type and dumpless mutants.

The down-regulation of *DIAP1* and *DIAP2* during stages 7 and 8 is also especially interesting. It was shown by Giorgi and Deri (1976) that most degenerating egg chambers in the *Drosophila* ovary were at developmental stages of 7 and 8. Loss of inhibitory factors during these stages may allow for regulation of egg production to occur through expression of a positive effector gene that results in apoptosis of superfluous or damaged egg chambers.

CONCLUSIONS

We have presented data that nurse cells are cleared from the egg chamber by apoptosis. Additionally, we have shown that DNA fragmentation in nurse cells is delayed in the presence of nurse cell cytoplasm. Finally, unlike apoptosis that occurs during embryogenesis, apoptotic cell death in oogenesis does not require genes within the *H99* deficiency. Further work should focus on the identity of genes or pathways that positively affect

the onset of apoptosis during oogenesis, and whether they are involved with initiating rapid cytoplasm transport. Additionally, further study of the *DIAP* genes is necessary to determine how they function in nurse cells. Analysis of apoptosis in the *Drosophila* ovary should provide a useful system for exploring the regulation of cell death in the germline.

Note added in proof

Recently McCall and Steller (Science 279:230, 1998) published a description of the ovarian phenotype for germline clones of the *Drosophila* caspase gene *dcp-1*. Egg chambers were defective in cytoplasm transport with specific defects in nurse cell nuclear envelop permeabilization and actin reorganization during stage 10.

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