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SHORT COMMUNICATION

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Apoptosis of nurse cells at the late stages of oogenesis of *Drosophila melanogaster*

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Abstract In *Drosophila* a remarkable feature of oogenesis is the regression of the nurse cells after dumping their cytoplasmic contents into the oocyte. We have studied the nature of this process at the late stages of egg chamber development. In egg chambers DAPI staining shows highly condensed chromatin from stage 12 and TUNEL labelling shows DNA fragmentation up to stage 14. Gel electrophoresis of the end-labelled DNA, extracted from isolated egg chambers at the same stages of development, shows a ladder typical of apoptotic nuclei. This provides evidence that, during *Drosophila* oogenesis, the nurse cells undergo apoptosis. Apoptotic nuclei have also been detected in dumping-defective egg chambers, indicating that the cytoplasmic depletion of nurse cells is concurrent with but apparently not the cause of the process.

Key words Apoptosis · Oogenesis · Nurse cells · *Drosophila melanogaster*

Introduction

Drosophila oogenesis begins when a germline stem cell divides asymmetrically so that one cell continues as a stem cell and the other becomes a cystoblast. Each cystoblast undergoes 4 mitotic divisions giving rise to a cluster of 16 cells surrounded by a follicular epithelium of mesodermal origin, forming the egg chamber. Incomplete cytokinesis during the 4 mitotic divisions results in 16 germline cells being interconnected by a series of cytoplasmic bridges or ring canals, through which cytoplasm can flow. Of the 16 cells, 1 develops into the oocyte, whilst the other 15 function as nurse cells. Reflecting the evolving morphology of the maturing egg chambers, oogenesis has been divided into 14 stages (King 1970). During oogenesis the majority of follicle cells rearrange into a columnar epithelium surrounding the growing oocyte, whilst a few follicle cells become squamous in shape and remain over the nurse cells. The highly polyploid nurse cells play a remarkable role in oocyte development providing it with the vast majority of cytoplasmic components and specific signals required for early embryonic development (King 1970; Spradling 1993; St Johnston and Nüsslein-Volhard 1992). During stages 1 to 10, a flux of cytoplasm gradually streams into the oocyte. Starting from stage 10B/11, a rapid transport of nurse cell cytoplasmic contents into oocyte, via the ring canals, occurs. A network of actin filaments polymerizes around the nurse cell nuclei, possibly preventing their movement during the cytoplasm flow (Cooley et al. 1992; Pfeifer et al. 1993); this process of massive transfer has been attributed to the myosin-based contraction of subcortical actin (Wheatley et al. 1995). Outcomes of cytoplasm transport into the oocyte are the regression of the nurse cell cluster and the doubling of oocyte volume, all occurring in less than 30 min. Following nurse cell breakdown, the nurse cell nuclei remain outside the oocyte and disappear at the final stages of the egg chamber development (King 1970; Spradling 1993).

We have studied the nature of the death process of the nurse cells after their function has been fulfilled, extending our analysis to the dumping-defective mutants *quail* and *singed*. This manuscript shows that, at the late stages of oogenesis, an apoptotic program is activated in the egg chamber of *Drosophila.*

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Materials and methods

Fly strains

All fly stocks were maintained under standard culturing conditions. Canton-S was used as the wild-type strain. The quail allele used was *qua^{HM14}* held in the stock *qua^{HM14}, cn, bw/CyO* (Mahajan-Miklos and Cooley 1994; kindly provided by L. Cooley). The singed allele used was *sn^{x2}* maintained in the stock Df(1)HA85/FM7c, *w^{a}, sn^{x2}, v^{Of}, g^{4}, B^{1}* (Cant et al 1994; provided by UMEÅ *Drosophila* stock centre).

DNA analysis

Four-day-old wild-type females of *D. melanogaster* were anaesthetized and dissected in the following buffer: 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM Hepes pH 6.9. Staged egg chambers were quickly collected and promptly frozen in liquid nitrogen. DNA was extracted as previously described by Pirrotta (1986) with the following modifications: after the homogenization step the samples were kept for 15 min at 65°C and for 15 min at 37°C with 100 µg/ml of proteinase K (Boehringer). The DNA was ethanol precipitated and resuspended in Tris-EDTA (TE: Sambrook et al. 1989). DNA samples were incubated for 1 h at 37°C with 30 µCi of α^{32}P-dideoxyATP (3000 Ci/mM, Amersham) and 25 units of terminal transferase in a final volume of 25 ml of 1 x terminal transferase buffer (Boehringer) and 2.5 mM $CoCl_2$. The DNA was precipitated by adding ammonium acetate to a final concentration of 2.5 M and 2 volumes of 98% ethanol. The DNA pellet was washed twice with 70% ethanol, dried briefly and resuspended in 10 µl of TE. The DNA was treated with RNase A and electrophoresed in 1.4% agarose gel in Tris-Borate-EDTA (TBE) running buffer (Sambrook et al. 1989). In the same gel were loaded different DNA molecular weight markers that were end-labelled with the same procedure described above. These samples were: Hind III-digested λ DNA, the molecular weight marker VI from Boehringer (pBR328/Bgl I + pBR328/Hinf I) and the DNA ladder obtained after micrococcal nuclease digestion of nuclei from adult Canton-S, performed as previously described (Worcel et al. 1983). After electrophoresis the gel was soaked for 30 min in 5% trichloracetic acid, dried and autoradiographed.

DAPI staining and TUNEL labelling

Isolated ovaries were fixed as previously described (Gargiulo et al. 1991), intensively washed with phosphate-buffered saline (PBS), and processed for DAPI (4′,6-diamidino-2-phenylindole) and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling) staining.

Ovaries were dehydrated in an ethanol series and embedded in Immuno-Bed (Polysciences). Three micrometer-thick sections, obtained with a Reicher-Jung Autocut microtome equipped with a glass knife, were stained for 10 min with DAPI 1 µg/ml in PBS and, after several washes with PBS, the sections were mounted in Fluoromount-G (Electron Microscopy Sciences) and examined by epifluorescence under a Zeiss Axioskop microscope.

For TUNEL labelling, ovaries were treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature with agitation on a rotating wheel. After three washes in PBS they were incubated, with agitation, for 30 min in 0.1% Triton X-100, 0.1% sodium citrate. The ovaries were washed twice with PBS and treated for 90 min at 37°C, with agitation, with 60 µM biotinylated dUTP, 0.2 U/µl of terminal transferase, 1 × terminal transferase buffer (Boehringer) and 2.5 mM $CoCl_2$. After three washes with PBS the ovaries were treated for 60 min, with agitation, with ABC reagent of the Vectastain Elite Kit (Vector). The ovaries were extensively washed with PBS and stained with DAB solution of the Vectastain Vector Kit (Vector). After a few washes in PBS, the ovaries were dissected in PBS, mounted in Fluoromount-G and viewed with a Zeiss Axioskop microscope equipped with Nomarski optics.

Results

Cell death in wild-type ovaries

Nuclear DNA condensation and fragmentation are characteristic features of apoptosis. We have analysed wild-type egg chambers by staining with the DAPI (4′-6-diamidino-2-phenylindole) technique that characterizes the chromatin structure, and the TUNEL technique that labels the 3′-end of DNA witnessing its fragmentation (Gavrieli et al. 1992). Figure 1 depicts the results of such experiments. Figure 1A shows a DAPI-treated section of an ovary where egg chambers at different stages are visible. DAPI stains a number of nuclei present in the cells at stage 12 of differentiation and, more intensely, one containing highly condensed chromatin. Fig. 1B, C shows the lower and higher magnification of a stage-12 egg chamber labelled with the TUNEL technique. At this stage TUNEL positivity starts to appear and two darkly stained nuclei are visible at the ventral side of the chamber. More nurse cells show staining at stage 13, when the typical apoptotic bodies can be detected (Fig. 1D, E). At stage 14 the apical region of the egg chamber is filled with TUNEL-positive DNA, indicating that all the nuclei have undergone apoptosis (Fig. 1F). It should be noticed that at the stages analysed no other cells but the nurse ones are TUNEL positive, indicating that the follicle cells are still alive and functional, in agreement with their role in the construction of the eggshell layers (King 1970; Mahowald and Kambysellis 1980; Spradling 1993).

DNA fragmentation into nucleosomal-size fragments is the diagnostic mark of apoptotic death (Wyllie et al. 1980). Thus we have analysed DNA from egg chambers isolated at relevant steps of oogenesis and from whole ovaries of wild-type females. The 3′ ends of the DNA were labelled by terminal transferase reaction using α^{32}P-dideoxyATP. Figure 2 shows the pattern of radioactive bands after agarose gel electrophoresis of the DNA. At stage 10, the egg chamber DNA does not show any fragmentation. At stages 12 and 13 a DNA ladder is clearly visible, with bands migrating to positions corresponding to 180 bp and multiples thereof. At stage 14 DNA fragmentation is more evident and up to seven DNA bands can be counted, all corresponding to oligonucleosomal DNA repeats. This pattern of fragmentation is identical to that of the DNA ladder generated by micrococcal nuclease digestion of chromatin, shown in the last lane of the figure. The DNA from the total ovary shows a pattern reflecting the presence of nurse cell nuclei at different functional stages.

The integrity of the DNA at stage 10 and its fragmentation at stages 12 and 13 coincide quite well with the

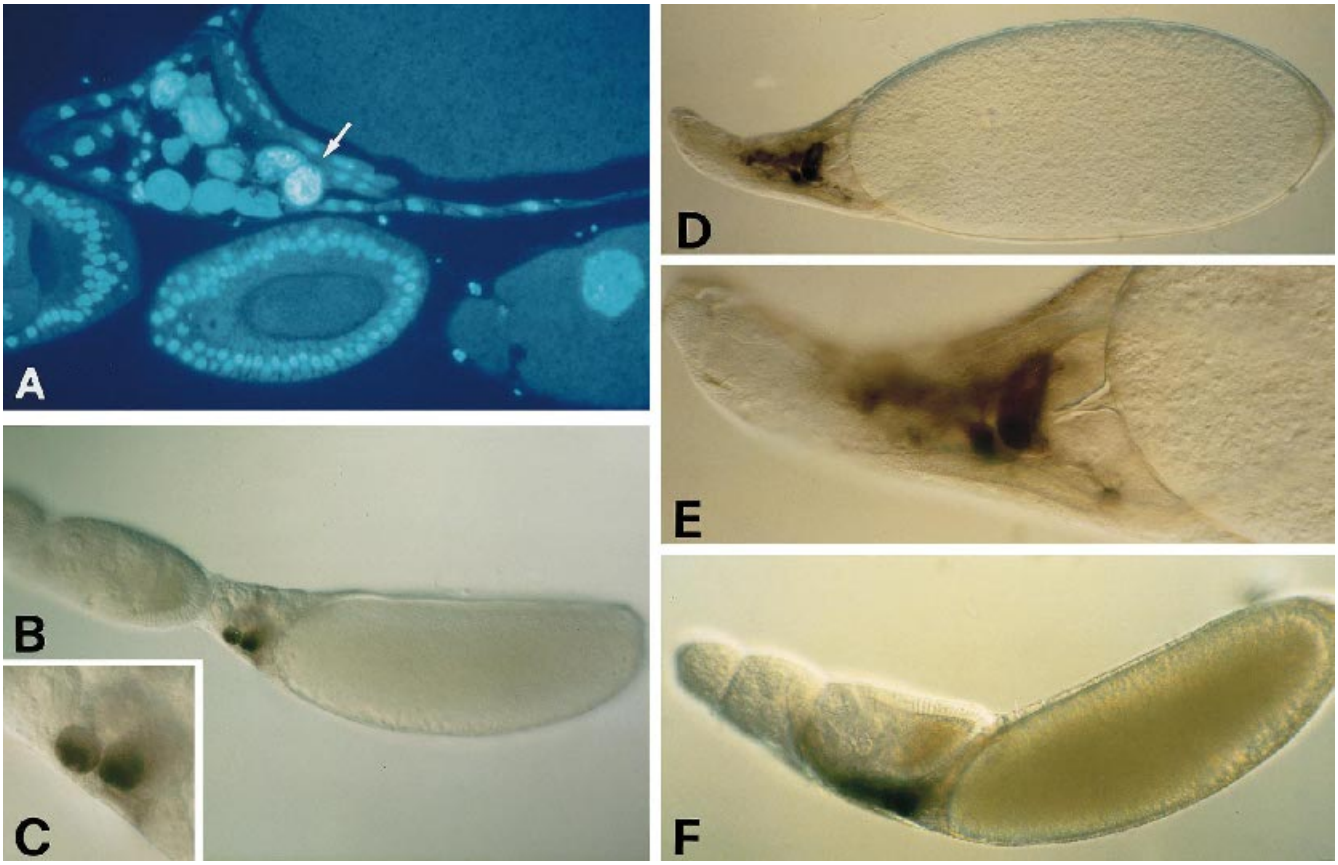

Fig. 1A–F In situ detection of cell death in wild-type egg chambers. **A** DAPI nuclear staining of an ovary section. The *arrow* points to one of the condensed nurse cell nuclei clearly visible in the stage-12 egg chamber. **B–F** TUNEL-labelled whole-mount egg chambers. **B** In the ventral side of the stage-12 egg chamber two positive nurse cell nuclei are visible. At higher magnification these nuclei show more intensely stained regions (**C**). **D** At stage 13 differently sized positive bodies are evident in the egg chamber tip. These stained bodies are better visible at higher magnification (**E**). **F** At stage 14 the egg chamber tip is filled by TUNEL-positive materials. All the egg chambers are oriented with the anterior end *to the left*

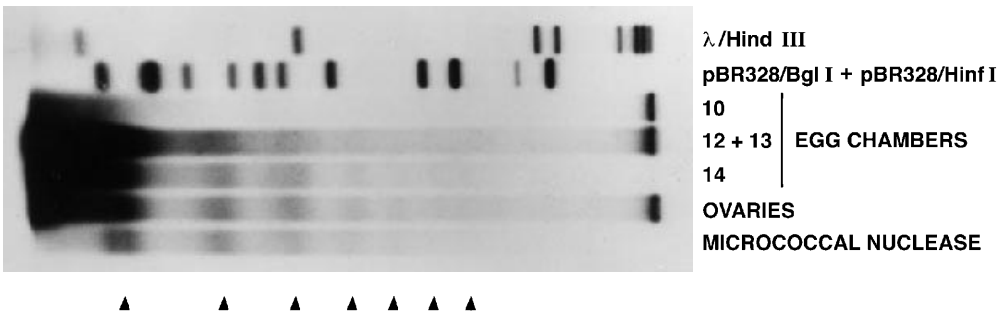

Fig. 2 Electrophoretic analysis of DNA fragmentation pattern. The arrowheads indicate the typical ladder pattern of internucleosomal DNA fragmentation

functionality of the nurse cells at the former stage and their regression at the later ones. For stage-14 egg chambers, although the follicle cells were still alive, no high molecular weight end-labelled DNA fragments were detected, probably because the amounts of DNA loaded in each lane were matched according to the counts of the α^{32}P-dideoxyATP incorporated. The expected band of high molecular weight DNA was scored when we analysed the DNA obtained from 250 egg chambers at stage 14 by agarose gel electrophoresis and ethidium bromide staining (data not shown).

We have observed a good correspondence between the morphological evidence obtained by the specific staining of the nuclei and the molecular evidence obtained by gel electrophoresis. In fact, the DNA ladder at stages 12 and 13, and 14 parallels the increasing TUNEL staining of the nuclei of the nurse cells at the same stages of differentiation. These data clearly show that the death

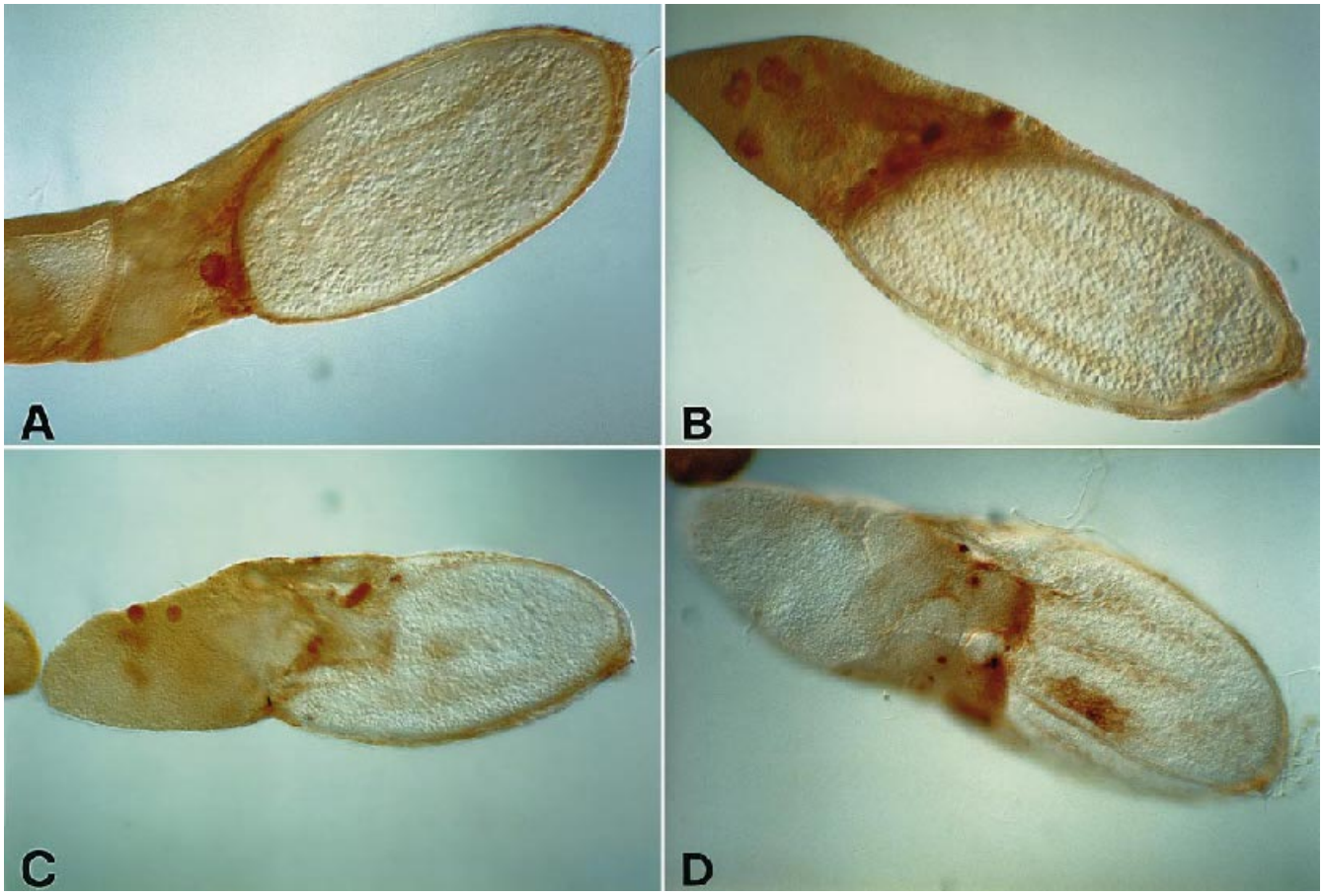

Fig. 3A–D In situ detection of cell death by TUNEL labelling. **A, B** *quail* egg chambers; **C, D** *singed* egg chambers. A few apoptotic nuclei are visible as darker brown spots in the nurse cell compartment (A, B and C). TUNEL positive signals, more easily scored in B and D, are also detected in the follicular epithelium. Egg chambers are oriented with the anterior end *to the left*

of the 15 nurse cells inside the egg chambers becomes apparent after the squeezing of the cytoplasm into the oocyte, starting with a few nuclei and progressively extending to the remaining ones.

Cell death in mutant ovaries

In order to understand if the dumping process per se could be the trigger of the apoptotic program, we have analysed egg chambers from *quail* and *singed* mutants, that show a nurse cell persistence caused by a defect of the massive cytoplasm transport (Cant et al. 1994; Mahajan-Miklos and Cooley 1994). These mutants are defective in the nurse cell cytoplasmic actin filament bundles that normally prevent the movement of their nuclei during the rapid cytoplasm transport (Cant et al. 1994; Mahajan-Miklos and Cooley 1994). The absence of these actin filaments allows the nurse cell nuclei to lodge into ring canals and block nurse cell cytoplasm transport.

In the resulting egg chambers, that contain oocytes smaller than normal, the follicle cells synthesize all the components of the egg shell including the respiratory appendages. In Fig. 3A, B the *quail* mutant analysed shows a fairly distinguishable apoptotic staining. Differing from the wild-type egg chambers at a comparable degree of maturation, not all of the nuclei appear to be in an apoptotic state (Fig. 3A). Some of the TUNEL-positive nuclei are found well surrounded by a relevant amount of cytoplasm. This indicates that the apoptotic process can be activated and proceeds even if the cytoplasm is retained in the nurse cells. DAPI staining of thin sections from *quail* ovaries shows a condensed state of chromatin comparable to the wild-type egg chambers (Fig. 4A, C). Whereas in the follicle cells of wild-type ovaries we could not detect TUNEL signals, in *quail* egg chambers from flies grown in the same conditions, the follicle cell nuclei appeared TUNEL-positive showing that apoptosis also occurs in the epithelium, and can be scored due to the prolonged retention of the mutant egg chambers. This suggests that a late apoptotic fate can also be expected for the wild-type follicle cells.

Apoptotic death is also detected in *singed* egg chambers where some condensed nuclei are shown by both TUNEL and DAPI stainings (Figs. 3C, 4B). As judged by DAPI staining, the nurse cell nuclei in *singed* egg chambers appear smaller than in the wild-type and high-

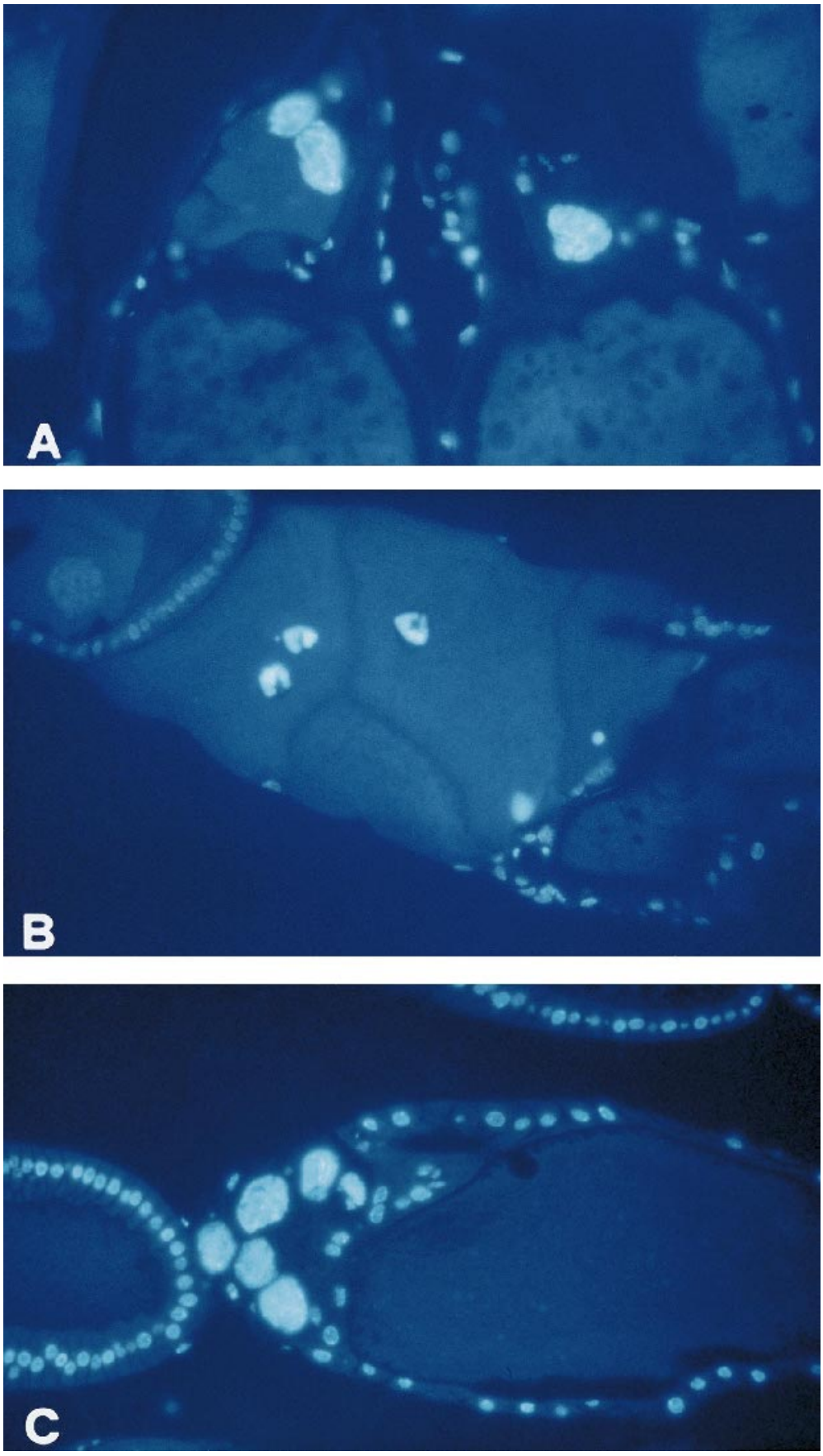

Fig. 4A–C DAPI nuclear staining of egg chambers section. **A** *quail*, **B** *singed*, **C** wild-type. Residual nurse cell nuclei stain intensely, showing highly condensed chromatin (A, B and C). In *singed* egg chambers nurse cell nuclei appear very reduced in size and two apoptotic bodies can be easily detected proximal to the oocyte

ly condensed (Fig. 4B), and in general they react poorly to TUNEL labelling (Fig. 3C).

Total DNA extracted from *quail* and *singed* mutant ovaries, analysed by agarose gel electrophoresis and ethidium bromide staining, showed a relevant amount of smeared DNA, significantly higher than that observed in wild-type ovaries, in addition to the typical oligonucleosomal DNA ladder (data not shown). This smeared DNA could indicate that not all of the dying cells go through a proper apoptotic program. Consistently, as shown in Fig. 3A–D, TUNEL reaction stains only a few nurse cell nuclei in *quail* and *singed* mutant egg chambers at a very advanced stage.

For both mutants these results suggest the activation of an apoptotic program in the nurse cells that is developmentally regulated and that appears to be delayed or hampered by the failure of cytoplasmic transfer.

Discussion

Cell death that occurs during the development of essentially all metazoan animals displays a characteristic ultrastructural morphology known as apoptosis, involving a series of distinct morphological changes which define this process (Kerr et al. 1972; Wyllie et al. 1980). Cells undergoing apoptosis shrink remarkably and show a condensed cytoplasm with normal-appearing organelles. Nuclear condensation, cleavage of DNA into nucleosomal-size fragments and apoptotic body formation are the morphological features of apoptosis. Molecular genetic studies in *Drosophila* have revealed the existence of apoptotic activators that play an important role in early development (for a comprehensive bibliography, see McCall and Steller 1997).

Giorgi and Deri (1976) described cell death in egg chambers undergoing abnormal development in *Drosophila*. Oogenesis in *Drosophila* is a highly organized process involving a variety of cell types that are removed after their function has been fulfilled and a mature egg is produced. Our results show that, during the normal development of the egg chamber, cell death by apoptosis is responsible for the removal of the exhausted nurse cells at the end of oogenesis. The analyses of the DNA extracted from total wild-type ovaries and staged egg chambers clearly show a DNA ladder with bands corresponding to oligonucleosomal DNA repeats. The high molecular weight DNA band visible in the lanes corresponding to egg chambers at stages 10, 12 and 13, and to the total ovary can be ascribed to the nurse cells which are still alive, and to the follicle cells. At stage 14, nurse cells should contain almost exclusively fragmented DNA whereas the follicle cells must still retain high molecular weight DNA, as detected by ethidium bromide staining after electrophoresis of the DNA extracted from 250 staged egg chambers.

In wild-type egg chambers the timing of nurse cell death follows the massive cytoplasm transport into the oocyte, but the finding of apoptotic nuclei in *quail* and *singed* egg chambers indicates that apoptosis is not initiated by cytoplasm dumping. Differences between wild-type and mutant ovaries through the course of apoptotic process were reflected at the molecular level, since electrophoresis of DNA from mutant egg chambers raised a smeared distribution superimposed on the apoptotic fragmentation pattern.

The defective oogenesis of the mutants analysed allowed us to recover old egg chambers showing TUNEL signal in the follicle epithelium. This suggests that at the end stage of oogenesis the apoptotic program should also be activated in the wild-type follicle cells. Therefore, in oogenesis, apoptosis appears to be a common fate for cells having fulfilled their role.

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Note added in proof

Recently McCall and Steller (Science 279: 230, 1998) published evidence about the requirement for DCP-1 caspase during *Drosophila* oogenesis. Morphological features of apoptosis in their *dcp-1*$^{-}$ germline clones parallel those of the dumpless mutants we analysed.