

Oocyte Development in *Hydra* Involves Selection from Competent Precursor Cells

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We have investigated oocyte development in *Hydra vulgaris*, a member of one of the oldest metazoan phyla. We show that oocyte determination involves a mechanism that establishes a subset of precursor interstitial cells competent to differentiate into oocytes. The oocyte is singled out from this subset and the competence of the remaining cells to become oocytes dramatically decreases as they adopt the alternative nurse cell fate. Progression through the nurse cell differentiation program requires the presence of the oocyte. When the oocyte is removed from the egg field, nurse cells abort their differentiation program, undergo apoptosis, and are phagocytosed and degraded by somatic epithelial cells. However, in the presence of the oocyte, nurse cells differentiate and enter an unusual apoptosis program where they are phagocytosed by the oocyte, but are not degraded. We show that the oocyte is able to induce this unusual apoptosis program in immature nurse cells that have not completed differentiation. A new model for oocyte development in *Hydra* is discussed. © 2000 Academic Press

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INTRODUCTION

Metazoan oocyte development strategies are diverse and specialized, although oocytes commonly differentiate from a pool of germ cells and grow in size by provision with nutrients synthesized by accessory cells called nurse cells. Nurse cells can be derived from germ-line cells or from somatic cells. Organisms in which nurse cells are derived from the germ line include the arthropod *Drosophila melanogaster* (reviewed in Williamson and Lehmann, 1996), the ctenophore *Bolinopsis microptera* (Harrison and Westfall, 1991), and the cnidarian *Hydra vulgaris* (Honegger *et al.*, 1989; Zihler, 1972). In organisms with germ-line-derived nurse cells, the germ cells must be partitioned into nurse cells and oocytes and the subsequent development of both

cell types must be coordinated to ensure proper oocyte growth. A lineage-based mechanism involving asymmetric germ cell divisions is thought to partition oocytes and nurse cells in *Drosophila* (de Cuevas and Spradling, 1998). However, little is known regarding the oocyte selection process or the role that the oocyte plays in regulating the nurse cell differentiation program in other animals.

To gain additional insight into the evolution of oogenic mechanisms used in metazoans, we were interested in studying oogenesis in the diploblast *Hydra*, a member of one of the oldest metazoan phyla (Collins, 1998; Schram and Ellis, 1994; Wainright *et al.*, 1993). In this simple organism, oocytes and nurse cells derive from a population of stem cells in the interstitial cell system that are committed to oogenesis, the egg-restricted stem cells (Littlefield, 1991). During oogenesis, these egg-restricted interstitial cells aggregate and proliferate rapidly beneath the ectodermal epithelium (Downing, 1909; Kleinenberg, 1872; Wager, 1909). Among the several thousand aggregated cells, one that is centrally located becomes the oocyte (Honegger *et al.*, 1989). The mechanism by which this cell is selected to become the oocyte is unknown. The remaining cells differentiate into nutrient-rich nurse cells, undergo apoptosis, and are subsequently phagocytosed by the oocyte where

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they persist throughout the completion of oogenesis and embryogenesis (Honegger *et al.*, 1989; Zihler, 1972).

We have investigated the oocyte determination mechanism and the role that the oocyte plays in regulating the nurse cell differentiation program in *Hydra*. As a foundation for our studies, oogenesis was divided into seven stages that are based on the morphology of the egg field, the area of oocyte development. Our results demonstrate the existence of a mechanism that singles out the cell that will become the oocyte from a subset of precursor cells that are competent to differentiate into oocytes. After the oocyte precursor is selected, the competence of the remaining cells to become oocytes dramatically decreases. In addition, we show that the presence of the oocyte is necessary for progression through the nurse cell differentiation program. When the oocyte is removed from the egg field, nurse cells abort their differentiation program, undergo apoptosis, and are phagocytosed and degraded by somatic epithelial cells.

MATERIALS AND METHODS

Hydra species and culture conditions. All analyses were carried out using a female strain of *H. vulgaris*, named AEP, which was derived from the PA1 strain isolated by Dr. Carolyn Teragawa (Martin *et al.*, 1997). Animals were maintained at 18°C in medium containing 1 mM CaCl₂, 1.5 mM NaHCO₃, 0.1 mM MgCl₂, 0.08 mM MgSO₄, and 0.03 mM KNO₃ and fed every 3 to 4 days with freshly hatched *Artemia salina* nauplii. Animals were starved during all experimental manipulations.

Grafting. Females possessing growing oocytes were cut transversely through the egg field at a position basal to the middle of the egg field whereas younger, *CnOtx*-positive females were cut transversely through the middle of the field (Fig. 4C). Grafts were constructed by threading nylon fishing line through the gastric cavity of the polyp pieces.

Acridine orange and toluidine blue stainings. Live polyps were incubated in 10 µg/ml acridine orange solution (Abrams *et al.*, 1993; Cikala *et al.*, 1999; Foley and Cooley, 1998) in hydra medium for 3 min in the dark. Following this incubation, polyps were rinsed in hydra medium four times and placed on slides in hydra medium. Samples were examined under a microscope with a fluorescent light source and photographed immediately. Whole animals were stained with toluidine blue essentially as described previously (Diehl and Burnett, 1964; Littlefield, 1985). Stained polyps were mounted in Euparal (Asco Laboratories) and photographed immediately.

Bromodeoxyuridine labeling and immunocytochemistry. To detect cells that are in the S phase of the cell cycle, a solution of 4.0 mM 5-bromo-2'-deoxyuridine (BrdU; Sigma) was injected into the gastric cavity of female polyps at various stages of oogenesis. After 1 h, the injected animals were relaxed in 2% urethane and fixed in 100% ethanol or alternatively macerated into a cellular suspension (David, 1973) and fixed in 70% ethanol. Whole polyps were incubated in 2 N HCl for 30 min, rinsed in PBS four times, and incubated with anti-BrdU antibody (Sigma). A fluorescein-conjugated secondary antibody was used for detection of bound primary antibody. Macerated preparations were stained with the same antibodies following attachment of the cells to subbed slides.

Whole and macerated polyps were mounted in 9:1 PBS:glycerol and photographed.

In situ hybridizations. Whole mount *in situ* hybridizations were carried out essentially as described previously (Grens *et al.*, 1995; Martinez *et al.*, 1997).

RESULTS

A Morphology-Based Staging Series for Hydra Oogenesis

Oogenesis in *H. vulgaris* was divided into seven stages based on morphological criteria (Fig. 1). We investigated the cellular architecture and events that occur during each stage to permit consistent identification and correlation of a particular stage with specific cellular and molecular processes. To visualize the aggregation of interstitial cells in the egg field, polyps were stained with toluidine blue. Since differentiating nurse cells do not cycle (Miller *et al.*, unpublished observation), pulse-labeling of cells with BrdU, a thymidine analog that is used to label cells in the S phase, made it possible to visualize the spatial distribution of cycling interstitial cells and differentiating nurse cells.

Stage 0. Prior to stage 1, interstitial cells accumulate in large numbers in the body column of sexual polyps (Littlefield, 1991). Before aggregation, these precursors cycle slowly relative to other interstitial cell types, progressing through the S phase only once every 2 to 3 days (Littlefield, 1991). Oogenesis begins with the aggregation of interstitial cells between the ectodermal epithelial cells of the body column.

Stage 1. Stage 1 polyps are characterized by the appearance of reddish pigment in the endodermal epithelial cells of the body column and a subtle swelling of the ectodermal layer over this region (Fig. 1, A1). This ectodermal swelling results from the aggregation and proliferation of interstitial cells (Fig. 1, B1). As stage 1 progresses, these interstitial cells continue to proliferate and aggregate. Toluidine blue stainings and BrdU pulse-labeling experiments demonstrate the increasing density of rapidly cycling interstitial cells in this area.

Stage 2. Stage 2 polyps possess an increased concentration of endodermal pigment and display an extensive swelling of the ectodermal layer due to the dense aggregation of interstitial cells (Fig. 1, A2). During this stage the aggregation reaches its maximum size, consisting of thousands of proliferating interstitial cells (Fig. 1, B2). The cells in the center of the egg field stop cycling and start differentiating into nurse cells (Fig. 1, C2). This differentiation process progresses radially from the center of the egg field. Nurse cell differentiation is characterized by an accumulation of lipid, glycogen, and yolk-like electron-dense material (Honegger *et al.*, 1989).

Stage 3. Stage 3 of oogenesis is marked by the first visible sign of the oocyte, which has the appearance of a large white spot located underneath the swollen ectoderm (Fig. 1, A3). Although most nurse cells have stopped cycling

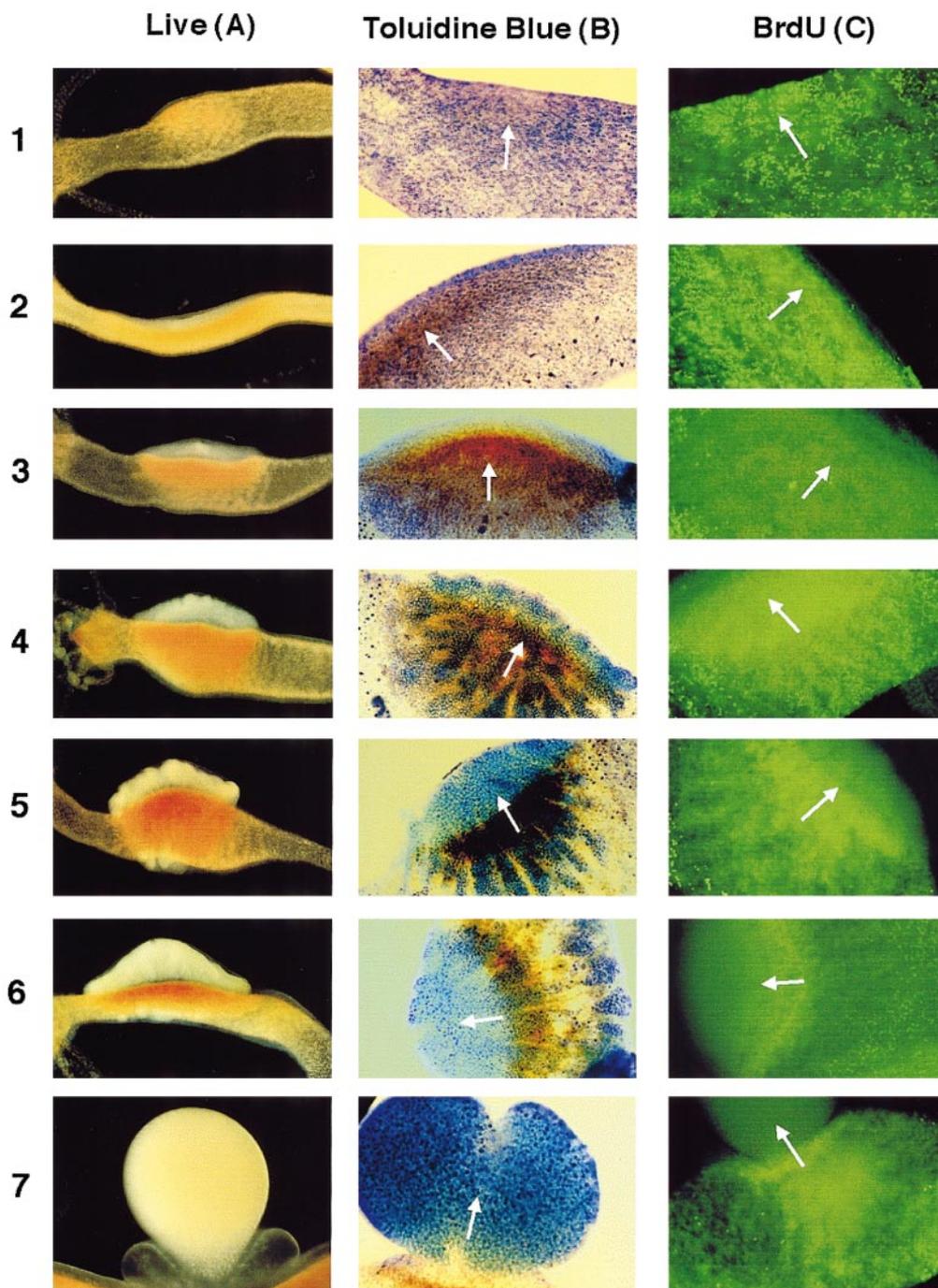


FIG. 1. Morphological and cytological features of the seven stages of *Hydra* oogenesis. (A) Dark-field photographs of live female polyps. (B) Toluidine blue-stained interstitial cells are light purple before oocyte phagocytosis and dark blue after phagocytosis. (C) BrdU pulse-labeling illustrates the pattern of interstitial cells in S phase of the cell cycle. Arrows point to the middle of the egg field (stages 1 and 2) or the middle of the oocyte (stages 3–7). Panel 7B shows a fertilized egg that has completed first cleavage.

by this stage, some cells near the periphery of the aggregation remain mitotic (Fig. 1, C3). At this time the oocyte starts increasing its volume due to the incorporation of

cytoplasmic fragments from the surrounding apoptotic nurse cells (Honegger *et al.*, 1989), which are densely packed.

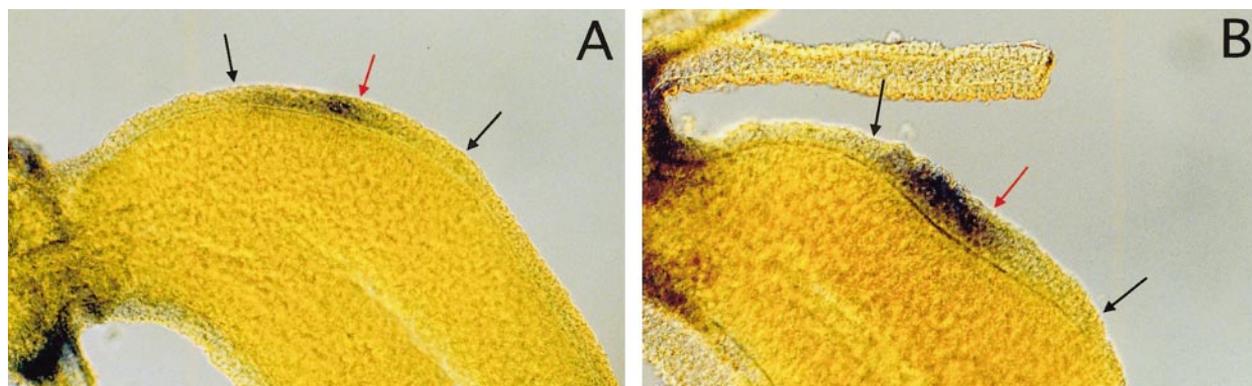


FIG. 2. The expression pattern of the *CnOtx* gene defines a subset of interstitial cells in the egg field during the time of oocyte determination. *CnOtx* transcripts appear in a thin oblong stripe of aggregating interstitial cells early in oogenesis (A), persist until the nurse cell growth stage is initiated (B), and then dissipate. Notice the apical bias of the expression pattern (a red arrow marks the center of the egg field, and black arrows mark the apical and basal borders of the field). The head is to the left in both panels. 100 \times .

Stage 4. During stage 4 the oocyte forms finger-like extensions (Honegger *et al.*, 1989) that engulf the surrounding nurse cells (Fig. 1, B4). These “pseudopods” often reach completely around the body column (Fig. 1, B4). Concurrent with phagocytosis, nurse cells develop morphological features of apoptosis and condense (Honegger *et al.*, 1989).

Stage 5. During stage 5, the oocyte begins retracting its pseudopods and initiates a massive morphological change (Fig. 1, A5 and B5). The distal ends of the pseudopods thicken relative to the proximal parts as the center of the oocyte starts to enlarge. As the fingerlike pseudopods retract, nurse cells are drawn toward the center of the oocyte.

Stage 6. The oocyte has fully retracted its extensions by the end of stage 6. The ectodermal layer continues to stretch as the oocyte assumes an oblong shape (Fig. 1, A6 and B6).

Stage 7. In the final stage of oogenesis, the oocyte breaks through the ectoderm and rests in a raised ring of retracted ectodermal tissue called the egg cup (Fig. 1, A7). This ectodermal retraction occurs in less than 15 min as the egg squeezes through the cleft. The oocyte completes meiosis and extrudes the polar body at its distal end. At this time, the oocyte, which is ready to be fertilized, is full of spherical, compacted nurse cells that consist of dense chromatin masses and remnants of endoplasmic reticulum (Honegger *et al.*, 1989). The diameter of a mature oocyte is approximately 0.5 mm. Due to the fragile nature of the mature oocyte, it was difficult to maintain them intact during mounting for microscopy. However, the egg cup can still be seen after the embryo has initiated cleavage (Fig. 1, B7).

The Expression Pattern of *CnOtx* Defines a Subset of Cells in the Egg Field

To date no genes have been reported whose expression is connected with oogenesis in *Hydra*. *CnOtx*, a *Hydra* ortho-

logue of the *Drosophila* homeobox gene *orthodenticle*, is dynamically expressed in epithelial cells during budding, the asexual form of reproduction in *Hydra*, and may have a role in specifying bud tissue (Smith *et al.*, 1999). RT-PCR expression analysis suggested that *CnOtx* is expressed during oogenesis (U. Technau, unpublished data). We therefore examined the spatio-temporal expression pattern of *CnOtx* in detail by *in situ* hybridization. In females undergoing oogenesis, *CnOtx* is also transiently expressed in a subset of aggregating interstitial cells within the egg field during stages 1 and 2 (Fig. 2). *CnOtx* RNA appears in a thin oblong patch of cells near the center of the egg field, but shifted slightly toward the apical end of the field (Fig. 2). This patch of staining, which consists of approximately 200 interstitial cells, dissipates after the interstitial cells in the middle of the egg field stop cycling and start to differentiate into nurse cells in the transition between stages 2 and 3. Since the oocyte becomes visible shortly after the disappearance of *CnOtx* expression in the egg field, we hypothesized that *CnOtx* expression might be related to the determination and/or differentiation of the oocyte.

More Than One Interstitial Cell in the Egg Field Is Competent to Differentiate into an Oocyte

The development of a single oocyte in the egg field could be the result of a single precursor cell that is already determined to become an oocyte prior to interstitial cell aggregation. Alternatively, the oocyte precursor could be selected from among many potential precursors competent for oocyte differentiation. In order to determine if multiple interstitial cells within the egg field are competent to differentiate into oocytes at the time of *CnOtx* expression, we took advantage of *Hydra*'s ability to regenerate. From a mass culture, females were selected and separated into one of two categories (*CnOtx*-positive or post-*CnOtx*) based on the morphology of the egg field. *CnOtx*-positive females

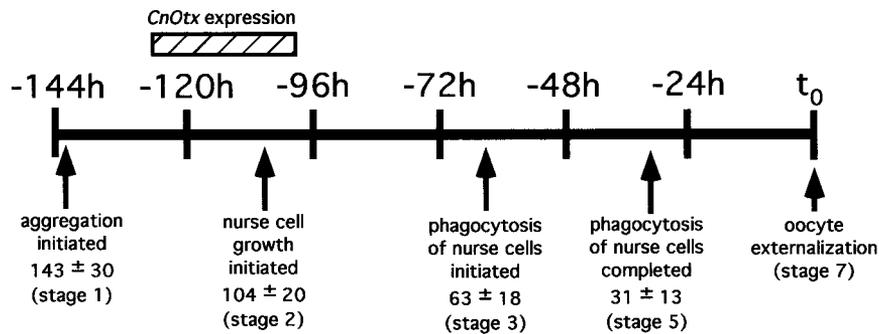


FIG. 3. The timing of oogenesis in starved females. Morphological characteristics were defined and linked to cellular events using several methods and histological stains. These morphological characteristics permit the consistent identification of stages. Twelve polyps were isolated during the first visible sign of interstitial cell aggregation and followed until oocyte externalization (t_0). The timing of *CnOtx* expression was correlated with these events based on the morphology of the egg field.

possess a colorless thickening of the epithelium above a region of pigmented endodermal cells (late stage 1 to early stage 2). Females in which *CnOtx* expression is no longer detected (post-*CnOtx*) are characterized by a milky coloring within the swollen epithelium marking the nurse cell growth stage (late stage 2) or later by the visible presence of the oocyte (stage 3). Figure 3 shows the timing of these events under our experimental conditions. Selected polyps were bisected through the middle of the interstitial cell aggregation, generating one side possessing the head and the upper half of the aggregation and the other side possessing the foot and the lower half of the aggregation (Fig. 4A). These bisected, regenerating polyps were observed carefully during the subsequent course of oogenesis. Delays in oocyte development were not observed in females that generated an oocyte. Of 144 bisected females, only 11 did not form an oocyte; these animals were not included in the analysis. The formation of functional oocytes was confirmed by fertilization and progression into the early cleavage stage of embryogenesis (data not shown).

If, for a given stage, a group of interstitial cells near the middle of the egg field is competent to differentiate into oocytes, then bisected females should produce an oocyte on each half significantly more often than uncut females (controls) produce two oocytes. Alternatively, if only one interstitial cell is competent to differentiate into the oocyte, then the proportion of bisected females that generate an oocyte on one half should be equal to the proportion generating one oocyte among uncut control polyps. Out of 70 control females selected randomly, 90% generated one oocyte per polyp, while only 10% formed two oocytes. In bisected *CnOtx*-positive females (Table 1A), oocytes were generated on both sides in over 39% of the bisections ($n = 71$) that generated at least one oocyte. In contrast, bisected post-*CnOtx* females (Table 1A) generated oocytes on both sides in only 6% of the bisections ($n = 62$) that generated

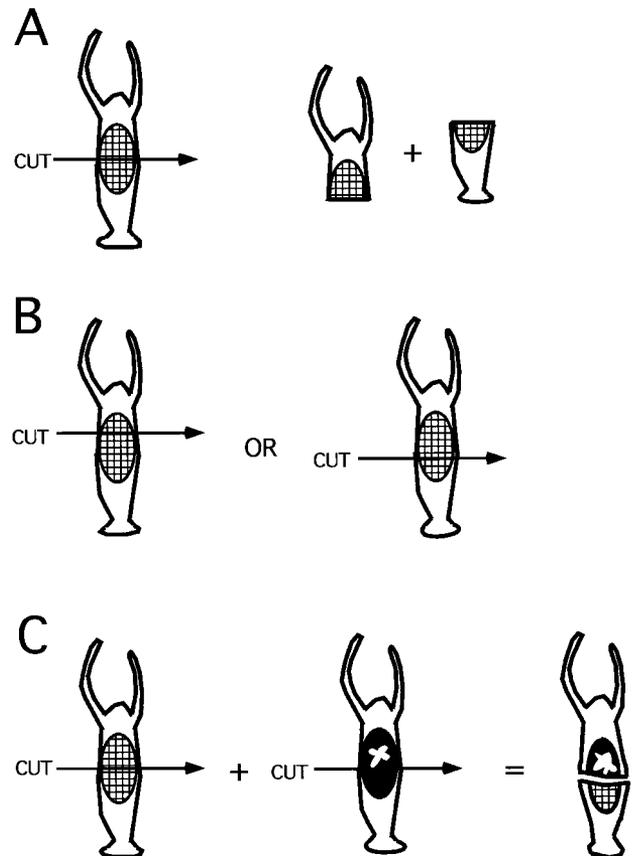
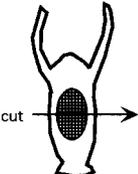
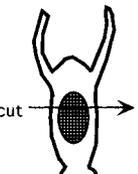
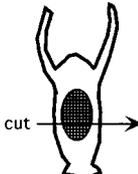


FIG. 4. Animal manipulations. In A, the egg field was bisected whereas in B, the egg field was dissected apically or basally generating two new egg fields containing one-third and two-thirds of the original field. In C, *CnOtx*-positive egg fields were bisected and grafted to dissected egg fields with growing oocytes (see text for details).

TABLE 1
Multiple Interstitial Cells Are Capable of Differentiating into the Oocyte

Experiment Description	Oocyte formation on head side only	Oocyte formation on foot side only	Oocyte formation on both sides	No oocyte formation
 <p>A</p> <p>CnOtx-positive</p> <p>Post-CnOtx</p>	25	18	28	5
 <p>B</p> <p>CnOtx-positive</p>	5	19	7	3
 <p>C</p> <p>CnOtx-positive</p>	30	0	1	2

Note. (A) Females were bisected through the middle of the egg field (see text for details). (B) *CnOtx*-positive females were dissected unequally into two fragments, one containing the head and one-third of the egg field and the other fragment containing the foot and the remaining two-thirds of the egg field. (C) The reciprocal experiment was also performed. The cross-hatched oval represents the egg field. See Results for statistical analyses.

at least one oocyte. A χ^2 test indicated that *CnOtx*-positive females generated oocytes on both sides a significantly higher number of times than the control group ($P < 0.01$) whereas post-*CnOtx*-positive females did not ($P > 0.1$). Therefore, *CnOtx*-positive females possess multiple interstitial cells which are capable of oocyte differentiation, while post-*CnOtx* females possess only one cell that is capable of oocyte differentiation. These results demonstrate that a region of competence exists during the time of *CnOtx* expression, but not after *CnOtx* expression has dissipated. In nonbisected polyps only one oocyte differen-

tiates in the middle of each egg field (10% of the time, multiple egg fields are present per polyp), although multiple interstitial cells are competent for oocyte differentiation. Hence, the oocyte must be singled out from these competent cells. After the oocyte enters meiotic prophase, the remaining interstitial cells enter the nurse cell differentiation pathway (Honegger *et al.*, 1989). Since post-*CnOtx*-positive polyps possess only one cell capable of oocyte differentiation, competent cells must lose their ability to differentiate into oocytes as they adopt the alternative nurse cell fate.

Not All Interstitial Cells in the Egg Field Are Equally Competent to Form an Oocyte in *CnOtx*-Positive Females

CnOtx expression is restricted to a thin stripe that is shifted apically in the egg field and does not extend into the basal third of the field (Fig. 2). If *CnOtx* expression defines the group of interstitial cells which are competent to differentiate into oocytes, then the experimentally determined region of competence should have the same asymmetric localization. To test this, we performed an experiment similar to the one previously described. However, instead of cutting the field of aggregating cells into equal-sized parts, the egg field was cut into two unequal parts, one part containing one-third of the aggregation and the other part containing two-thirds of it (Fig. 4B). If a group of competent cells is shifted apically in the egg field and does not extend into the basal third, then the basal third of the egg field should not be able to generate an oocyte since this region does not contain competent cells. Alternatively, the apical third of the egg field should be able to generate an oocyte. In agreement with this hypothesis, females dissected apically (Table 1B) generated an oocyte in the apical third of the egg field nearly 39% ($n = 31$) of the time ($P > 0.1$). Interestingly, these oocytes were smaller due to the decrease in the number of nurse cells, but were still able to be fertilized and undergo cleavage. In females dissected basally (Table 1C), an oocyte was generated in the basal third only 3% ($n = 31$) of the time, significantly less than would be expected from an equal distribution of competent cells ($P < 0.01$). This demonstrates that our experimentally determined region of competence is apically shifted within the egg field. In summary, the spatio-temporal expression pattern of *CnOtx* correlates with the spatio-temporal pattern of competence to form an oocyte.

The Presence of the Oocyte Is Necessary for Progression through the Nurse Cell Differentiation Program

The field of competence for oocyte development as demonstrated above comprises only a subregion of the egg field, yet aggregated interstitial cells from the periphery (outside the region of competence) also differentiate into nurse cells. This leaves two alternative explanations: (i) nurse cell differentiation is the default program of the aggregated interstitial cells, or (ii) aggregated interstitial cells require a specific signal to initiate the nurse cell differentiation program. Since differentiation of nurse cells moves radially from the center of the egg field after oocyte determination, the oocyte itself is a candidate for the source of such a signal. As a first step in testing this hypothesis, we wanted to examine the fate of aggregating interstitial cells that were never in the presence of an oocyte. If, prior to oocyte selection, interstitial cells which are not competent for oocyte differentiation are separated from the egg field, these noncompetent interstitial cells would not receive hypothetical signals from an oocyte since oocyte differentiation

within the separated aggregation is not possible. Therefore, these noncompetent interstitial cells should not complete the nurse cell differentiation program. To test this idea, we used two different experiments to isolate subsets of interstitial cells which are not competent for oocyte differentiation from *CnOtx*-positive egg fields.

Our previous results showed that the lower third of an egg field in *CnOtx*-positive females is not competent to generate an oocyte (Table 1C). In order to analyze the effect of the presence or absence of the oocyte on nurse cell differentiation we used acridine orange (AO) staining. AO labels apoptotic cells in live animals (Abrams et al., 1993; Cikala et al., 1999; Foley and Cooley, 1998; Gumienny et al., 1999) and is, therefore, a useful indicator of progression through the nurse cell differentiation program. Since differentiated nurse cells undergoing apoptosis have a different morphology than other interstitial cells undergoing apoptosis (Fig. 6), we can also determine to what extent cells have differentiated into nurse cells prior to apoptosis. Females at the *CnOtx*-positive stage of oogenesis were dissected through the lower third of the egg field and stained with acridine orange after 12, 24, and 48 h. On the head side of each dissection, which contained interstitial cells competent for oocyte differentiation (control), the nurse cell differentiation program proceeded normally (Fig. 5A). However, on the foot side (Fig. 5B), which contained no interstitial cells competent for oocyte differentiation, nearly all aggregating interstitial cells completed apoptosis and were phagocytosed and degraded by somatic epithelial cells within 48 h after dissection. These apoptotic interstitial cells did not complete the nurse cell differentiation program and instead entered apoptosis prematurely and were degraded. Morphologically, they resemble apoptotic interstitial cells from HU-treated polyps (Fig. 6B) and do not possess the morphology typical of differentiated nurse cells (Fig. 6C).

The above result was confirmed by an alternative experiment. Instead of basally dissecting polyps, we made incisions through the epithelium of *CnOtx*-positive egg fields which completely split a region of the aggregation, but kept the polyp in one piece. Thus, we were able to isolate small regions of the egg field lacking interstitial cells competent for oocyte differentiation. In this situation, the interstitial cells within the isolated regions completed apoptosis and were degraded by epithelial cells within approximately 36 h (data not shown). These two experiments show that in the absence of an oocyte, interstitial cells do not complete the nurse cell differentiation program and instead enter apoptosis prematurely and are degraded.

Next, we wanted to remove the oocyte from the egg field to observe the effect on the nurse cell differentiation program. Mid-stage 2 polyps and early stage 3 polyps were isolated based on the morphology of the egg field and bisected. Subsets of the bisected polyps were stained with AO 24, 48, and 72 h following bisection. In each bisection, the side possessing the oocyte developed normally and served as a control (Fig. 5C). In sides where the oocyte was

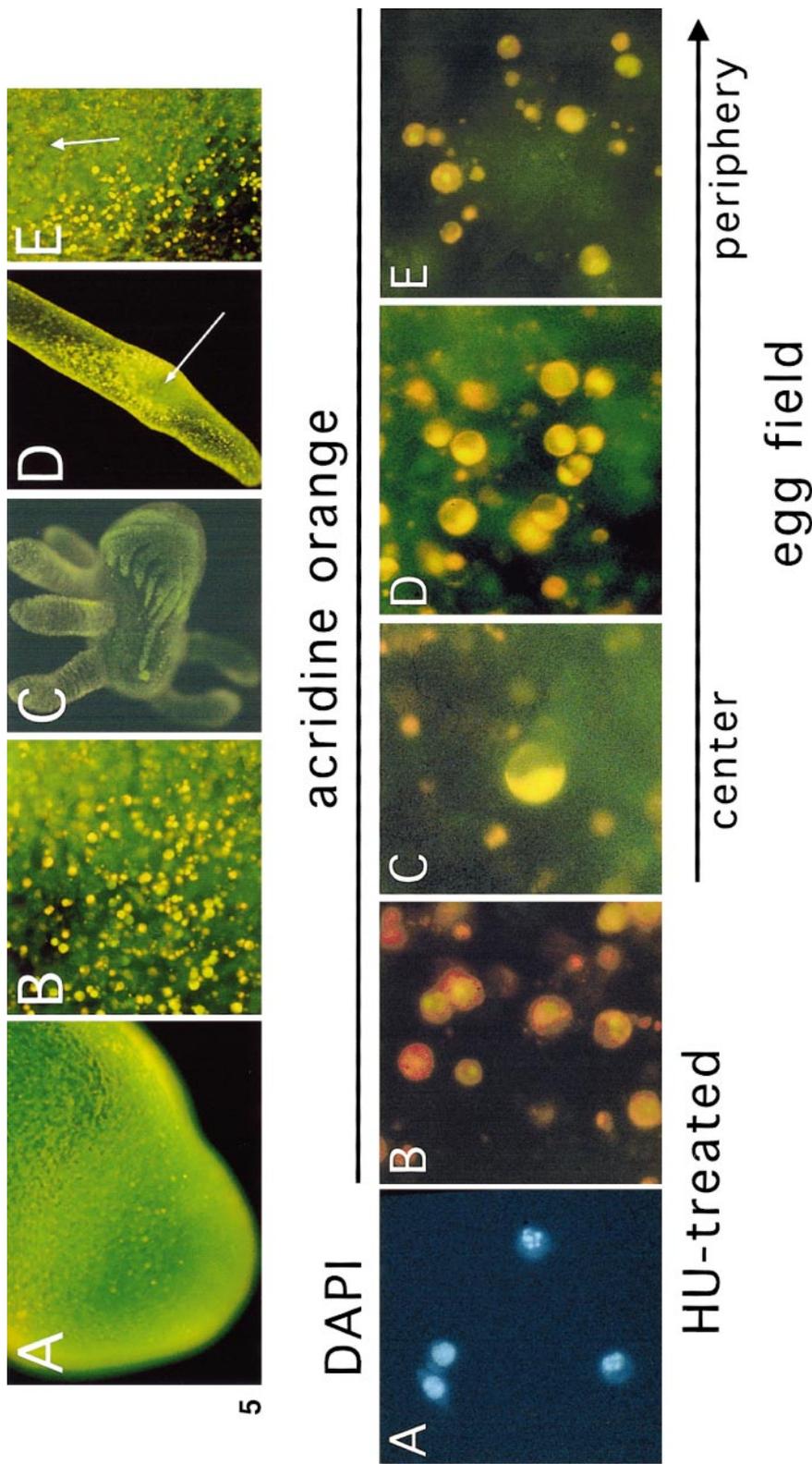


FIG. 5. AO stainings of live polyps from dissected egg fields. (A) The head side of an egg field 24 h after the dissection of a CnOtx-positive female. All nurse cells on this side, which contained interstitial cells competent for oocyte differentiation, are differentiating and are not brightly labeled with AO. 100 \times . (B) Close-up of apoptotic interstitial cells 24 h after the dissection of a CnOtx-positive female. This separated aggregation lacked interstitial cells which are competent for oocyte differentiation. 200 \times . (C) An egg field possessing the oocyte 48 h after the bisection of a post-CnOtx female. The pseudopods of the oocyte are visible. All nurse cells have differentiated and have been phagocytosed by the oocyte where they are labeled bright green by AO. 40 \times . (D) Degrating egg field lacking the oocyte 48 h after the bisection of a post-CnOtx female. An arrow points to the middle of the egg field, which contains nonapoptotic nurse cells that are not labeled with AO. The peripheral cells stain yellow/red with AO indicating that they are apoptotic. The size of the egg field has been greatly reduced due to apoptosis. 40 \times . (E) Close-up of apoptotic nurse cells from a degenerating egg field lacking the oocyte 48 h after the bisection of a post-CnOtx female. An arrow points to the middle of the egg field, which contains differentiating nurse cells that are not labeled with AO. 200 \times .

FIG. 6. Interstitial cell morphologies. (A) Interstitial cells from an HU-treated polyp stained with DAPI. Note the condensed chromatin indicative of apoptosis in the lower two cells. (B) A live AO-stained, HU-treated polyp illustrating the morphology of interstitial cells undergoing apoptosis. 400 \times . (C-E) AO-stained nurse cells in the egg field from bisected post-CnOtx females where the oocyte was removed. The morphology of a fully differentiated apoptotic nurse cell near the center of the field is shown in C. 400 \times . Apoptotic nurse cell precursors near the periphery of the field, which have not differentiated (E) have the morphology of apoptotic interstitial cells from HU-treated polyps (B). (D) Cells located in the central region of the field between C and E.

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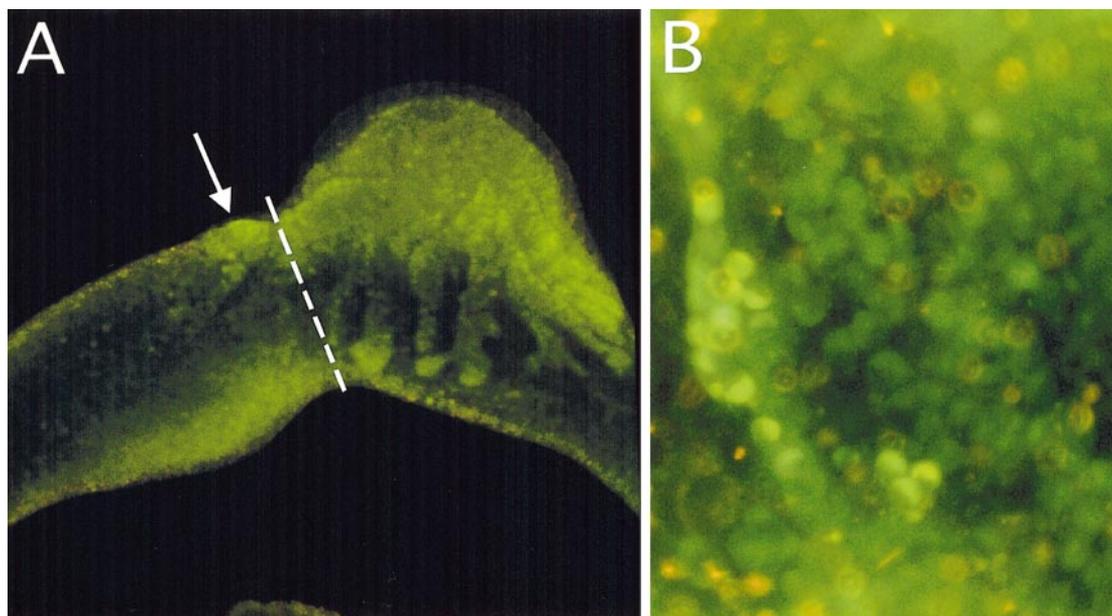


FIG. 7. The oocyte is able to induce apoptosis in immature nurse cells. Grafts were constructed as shown in Fig. 4C and described in the text, and the animals were stained with AO 24 h after grafting. (A) Pseudopods (indicated by the arrow) from the growing oocyte (to the right of the dashed line, which marks the graft junction) have invaded the younger egg field (to the left of the dashed line) and phagocytosed the immature nurse cells. 40 \times . (B) During phagocytosis of these immature nurse cells by an oocyte pseudopod, cellular compaction occurs (indicated by bright green staining with AO). 200 \times .

removed, nurse cells aborted their differentiation program, entered apoptosis prematurely, and were degraded by somatic epithelial cells (Figs. 5D and 5E). Two phenomena were observed in these bisected females. First, nurse cells located near the periphery of the egg field initiated apoptosis before nurse cells located near the center of the egg field (Figs. 5D and 5E). The egg fields progressively degenerated as more central nurse cells completed apoptosis. Second, nurse cells located near the center of the egg field initiated apoptosis with a differentiated nurse cell morphology (Fig. 6C). In contrast, nurse cells located near the periphery (Fig. 6E) completed apoptosis with a morphology similar to that of apoptotic interstitial cells in HU-treated polyps (Fig. 6B), indicating that they had not completed differentiation. Females bisected early in stage 3 contained higher densities of differentiated nurse cells relative to females bisected at mid-stage 2 (data not shown). Interestingly, the formation of pseudopods was often initiated in sides lacking an oocyte, but was localized to the most central region of the egg field with a very irregular pattern. Nurse cell apoptotic bodies were observed in the pseudopods, but the pseudopods failed to grow. Females bisected during stage 3 initiated this process in nearly all of the bisections, whereas females bisected within approximately 12 h after determination initiated this process in less than one-third of the bisections.

These results show that the presence of the oocyte is necessary for continued progression through the nurse cell

differentiation program. The last step in this program is a form of apoptosis where the nurse cells are phagocytosed by the oocyte, but they are not degraded. When the oocyte is removed from the egg field, the nurse cells abort this differentiation program and enter and complete apoptosis prematurely, suggesting that the oocyte promotes the survival and differentiation of nurse cells.

The Oocyte Induces the Apoptosis of Immature Nurse Cells

We hypothesized that the oocyte was promoting nurse cell survival and differentiation only to later induce apoptosis to facilitate oocyte growth. To test this idea, grafts were created that fused two dissected egg fields together at different stages in their development (Fig. 4C). Egg fields with growing oocytes possessing small pseudopods (stage 3–4) were grafted to *CnOtx*-positive egg fields (stage 2), allowed to heal for 24 h, and stained with AO. Although in most cases the two egg fields remained separated, in 6 out of 30 grafts, pseudopods from the growing oocyte invaded the periphery of the younger egg field and phagocytosed the immature nurse cells (Fig. 7B). During phagocytosis, these immature nurse cells condensed, forming compact, spherical corpses identical to those phagocytosed from the mature field. In 3 of the 6 grafts, the egg fields physically merged and extensive invasion and phagocytosis was observed (Fig.

7A). In all 6 cases, apoptosis of immature interstitial cells occurred concurrent with pseudopod phagocytosis.

DISCUSSION

Previous investigations of oogenesis in *Hydra* (Aizenshtadt, 1975, 1978; Downing, 1909; Kleinenberg, 1872; Tardent, 1985; Wager, 1909) established that oocytes and nurse cells derive from interstitial cells residing beneath the ectodermal epithelium. Littlefield (1991) showed that oocytes and nurse cells derive from a subpopulation of egg-restricted stem cells in the interstitial cell lineage. We show that during oogenesis the oocyte is selected from a subset of interstitial cells that are competent to differentiate into oocytes and that the presence of the oocyte is necessary for progression through the nurse cell differentiation program.

Oocyte Determination in Hydra

Most *Hydra* species reproduce asexually by budding under normal laboratory conditions. Gametogenesis can be induced in the *H. vulgaris* AEP1 strain that we used by reducing the frequency of feeding. Under this condition, large numbers of interstitial cells aggregate and proliferate at the onset of oogenesis in *Hydra*. Our results demonstrate the existence of a mechanism acting on this aggregation of cells that (1) establishes a subset of cells competent to differentiate into an oocyte and that (2) singles out the oocyte from this subset. Although the role of *CnOtx* in the oocyte determination mechanism is not known, its spatio-temporal expression pattern defines a subset of cells that is distributed in a pattern that coincides with our experimentally determined region of competence. *CnOtx* may be involved in the establishment and/or maintenance of a subset of interstitial cells within the egg field that are capable of differentiating into oocytes. Ultimately, the oocyte is singled out from this subset.

Although the mechanism of oocyte determination in *Hydra* does not resemble the mechanism used in merostic insects (de Cuevas and Spradling, 1998; Klag and Bilinski, 1994; Szklarzewicz and Bilinski, 1995), it resembles a situation observed in the *Drosophila* peripheral nervous system where sensory organ precursor cells (SOPs) are specified. Here, regions of the embryonic epithelium acquire neural competence and express proneural genes such as *achaete* and *scute*. One centrally located cell is singled out to be the SOP in a process that is mediated by cell-cell interactions involving Notch and Delta (reviewed in Greenwald, 1998; Jan and Jan, 1994; Schweisguth *et al.*, 1996). The SOP then divides and differentiates into the sensory organ while all other competent cells adopt the alternative epithelial cell fate (reviewed in Greenwald, 1998; Jan and Jan, 1994; Schweisguth *et al.*, 1996). Although no expression of *Cnash*, the *Hydra* homologue of *achaete/scute* (Grens *et al.*, 1995) could be detected during oogenesis (data

not shown) it will be interesting to see if cell fate specification during oogenesis in *Hydra* involves Notch/Delta signaling.

The Regulation of Nurse Cell Differentiation

After the oocyte is selected, the remaining interstitial cells differentiate into nurse cells and undergo apoptosis (Honegger *et al.*, 1989; Zihler, 1972). We showed that aggregated interstitial cells do not differentiate into nurse cells in the absence of the oocyte. This rules out the possibility that nurse cell differentiation is the default program of aggregated interstitial cells and argues for a signal being required for nurse cell differentiation. Two results strongly suggest that the oocyte is the source of the signal: (i) Following determination of the oocyte, aggregated interstitial cells stop cycling and enter the nurse cell differentiation pathway in a concentric wave starting adjacent to the oocyte (Fig. 1). (ii) In the absence of the oocyte, interstitial cells do not differentiate into nurse cells (Fig. 5). Further, we show that the oocyte is able to induce apoptosis even in immature nurse cells that have not completed differentiation (Fig. 7). This shows that a fully differentiated state is not a precondition for nurse cell apoptosis. Hence, the oocyte is responsible for maintaining interstitial cell survival within the egg field only to later induce apoptosis in the nurse cells to facilitate oocyte growth.

Interestingly, nurse cells can undergo two forms of apoptosis depending on the presence or absence of the oocyte. In the presence of the oocyte, nurse cells enter the apoptotic program and are phagocytosed by the oocyte's pseudopods, but they are not degraded and their corpses persist within the oocyte and developing embryo. In the absence of the oocyte or a signal from the oocyte, nurse cells abort their differentiation program, complete apoptosis prematurely, undergo DNA fragmentation, and are phagocytosed and degraded by surrounding epithelial cells. In keeping with these findings, nurse cells phagocytosed by the oocyte are TUNEL-negative, while peripheral nurse cells not phagocytosed by the oocyte are TUNEL-positive (U. Technau, unpublished observations). Our results suggest that nurse cell apoptosis consists of two distinct steps. In the first step, the cells initiate apoptosis as partially or fully differentiated nurse cells. Subsequent phagocytosis of the nurse cells by the oocyte results in suspension of the apoptosis program until hatching of the young polyp. Upon hatching, the second and final phase of the apoptotic program ensues, involving further changes in the DNA (as indicated by a change in AO staining color from green to orange) and phagocytosis of the nurse cells by the endodermal cells of the polyp (Miller *et al.*, unpublished observations).

A Model for Oogenesis in Hydra

Our results and the results from previous studies suggest a model for oogenesis in *Hydra* which is shown in Fig. 8.

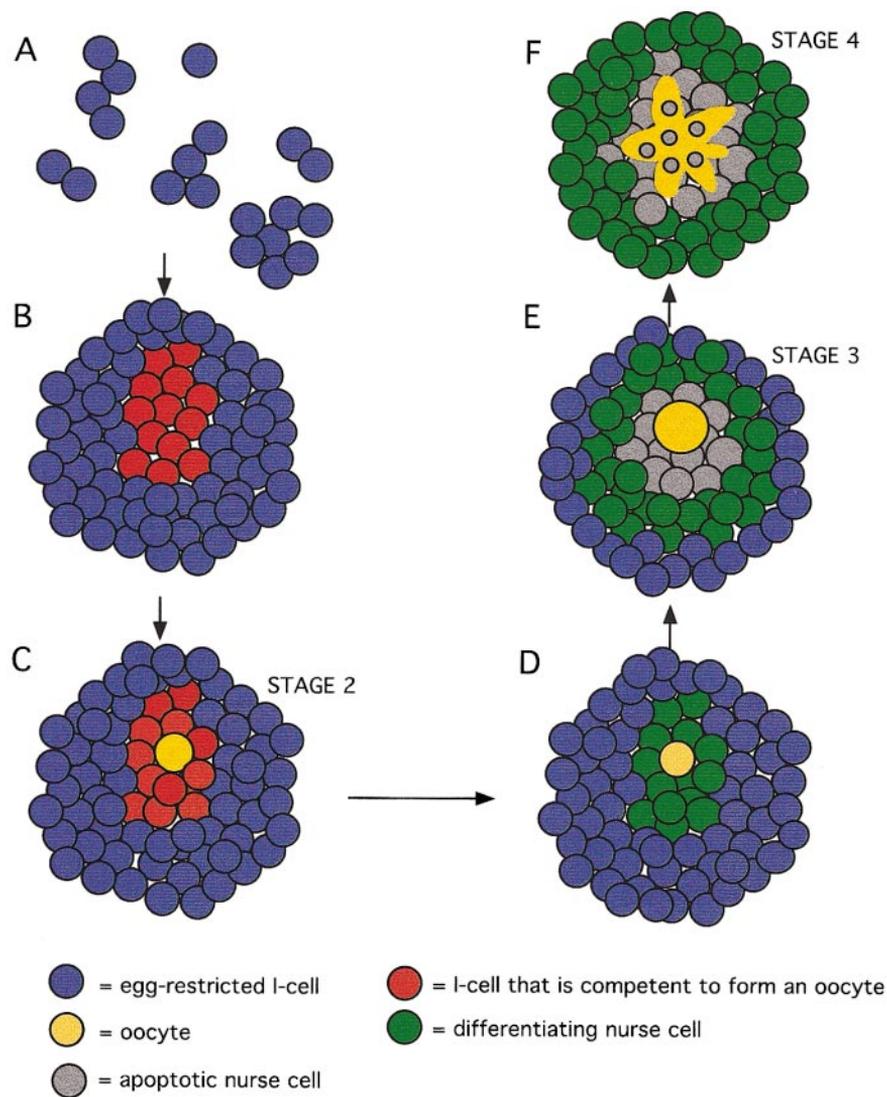


FIG. 8. Model for oocyte determination in *Hydra*. The details of the model are described in the text.

Egg-restricted interstitial cells aggregate and proliferate (Figs. 8A and 8B) at the onset of oogenesis (Littlefield, 1991). A subset of cells located near the center of the egg field (but slightly biased toward the apical end) is competent to differentiate into oocytes (competent cells could arise anytime during 8A–8B). The oocyte is selected from this subset of cells, while the surrounding cells lose the ability to become oocytes and adopt the alternative nurse cell fate (Figs. 8C and 8D). The presence of the oocyte is necessary for continued progression through the nurse cell differentiation program, which is initiated near the middle of the aggregation (Figs. 8D and 8E). Later, the oocyte induces apoptosis in the surrounding nurse cells and phagocytoses them (Figs. 8E and 8F) resulting in massive oocyte growth.

Phagocytosed nurse cells condense (Fig. 8F), but are not degraded until days after hatching.

Evolution of Oogenic Mechanisms

Although oocyte development has been studied in considerable detail in a few model systems, we still know very little about the mechanisms underlying oogenesis in many animals. In the absence of such information it is difficult to know to what extent oocyte development in various animal phyla represents modification of an ancestral program. An examination of the available information on oogenesis in diverse animal phyla presents several intriguing similarities. One oocyte development strategy that is particularly

widespread involves the use of nurse cells to facilitate oocyte growth (de Cuevas and Spradling, 1998; Eckelbarger, 1982; Gumienny *et al.*, 1999; Harrison and Westfall, 1991; Honegger *et al.*, 1989). The existence of nurse cells in a cnidarian demonstrates that this type of oocyte development was invented early in metazoan evolution. Although detailed information regarding the mechanisms of nurse development is available only for *Drosophila*, some similarities in nurse cell development are present in diverse animals. In many species of sponges, ctenophores, cnidarians, and insects the oocyte is located in the middle of a germ cell aggregation (Harrison and Westfall, 1991). The presence of cytoplasmic bridges between germ cells during oogenesis is another common feature. In *Hydra*, such bridges are present, but they are lost during nurse cell differentiation (Honegger *et al.*, 1989; Littlefield, 1991). Finally, a striking similarity among *Drosophila* (Foley and Cooley, 1998; McCall and Steller, 1998), *C. elegans* (Gumienny *et al.*, 1999), and *Hydra* is the presence of a germ cell apoptosis program (Honegger *et al.*, 1989; Zihler, 1972). In both *Drosophila* and *Hydra*, germ-line-derived nurse cells enter apoptosis but are prevented from complete degradation until the transfer of nutrients, organelles, and specific mRNAs and proteins (in *Drosophila*) or the hatching of the embryo (in *Hydra*) is completed. The extent to which the similarities we see in the oogenic programs of *Hydra* and other animals have their origins in an ancestral program should become clear as more detailed studies of oogenesis in diverse animals are carried out.

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