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Yolk sphere formation is initiated in oocytes before development of patency in follicles of the moth, *Plodia interpunctella*

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Abstract. We describe a provitellogenic stage, a previously unrecognized stage of follicle development in moths, and show that oocytes begin yolk sphere formation prior to the development of patency by the follicular epithelium. The vitellogenic activities of follicles from pharate adult female *Plodia interpunctella* (Hübner) were determined by visualizing the subunits of vitellin (YP1 and YP3) and the follicular epithelium yolk protein (YP2 and YP4) using monospecific antisera to each subunit to immunolabel whole-mounted ovaries or ultrathin sections. At 92 h after pupation, yolk spheres that contained only YP2 began to proliferate in the oocytes. The inter-follicular epithelial cell spaces were closed at 92 h making vitellogenin inaccessible to the oocyte, and consequently, the vitellin subunits were not observed in the yolk spheres. YP2 uptake most likely occurred across the brush border from the follicular epithelial cells to the oocyte at this time. At 105 h, the inter-follicular epithelial cell spaces appeared closed yet trace amounts of labeling for vitellin were observed in the spaces and also in the yolk spheres along with YP2. Equivalent labeling for all four YPs in yolk spheres was finally observed at 112 h after pupation when the follicular epithelium had become patent. These data indicate that the provitellogenic stage is an extended transition period between the previtellogenic and vitellogenic stages that lasts for approximately 13 h, and it is marked at the beginning by YP2 yolk sphere formation in the oocyte and at the end by patency in the follicular epithelium.

Key words: Vitellogenesis – Immunofluorescent staining – Immunogold labeling – Yolk proteins – Oocyte development

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Introduction

As a follicle of an insect becomes vitellogenic, the oocyte must assemble sufficient cellular machinery and gain access to the necessary precursors to support the subsequent rapid accumulation of yolk proteins and formation of yolk spheres (cf. Telfer 1965; Engelmann 1979; Hagedorn and Kunkel 1979; Telfer et al. 1982; Kunkel and Nordin 1985; Raikhel and Dhadiallia 1992). In previtellogenic oocytes of the anautogenous mosquito, *Aedes aegypti*, the production of a specialized cortex, which contains numerous coated pits, vesicles, and endosomes in or near the brush border, is necessary to support the rapid uptake of yolk proteins following the onset of vitellogenesis after a blood meal (Raikhel and Lea 1985). In order for the oocyte to gain access to vitellogenins, which are produced in the fat body and transported to the ovary in the hemolymph, the follicular epithelium must develop patency, i.e., large intercellular spaces (Pratt and Davey 1972). Patency, thus, permits the movement of vitellogenins from the hemolymph to the brush border of the oolemma (Telfer 1961). In the bug *Rhodius prolixus*, the development of patency and, therefore, the initiation of yolk uptake is controlled in maturing follicles by increased hemolymph titers of juvenile hormone (Davey 1981). However, in the giant silkmoth, *Hyalophora cecropia*, the development of patency in the follicular epithelium has not been associated with any specific regulatory mechanism (Telfer et al. 1982).

The initiation of vitellogenesis in the pharate adult Indianmeal moth, *Plodia interpunctella* (Hübner), has provided a system for examining follicle development in moths. Our previous work showed that pharate adult development was synchronous and correlated with photoperiod (Zimowska et al. 1991). The availability of synchronously developing pharate adults has resulted in an accurate temporal framework for follicle maturation (Zimowska et al. 1991), vitellogenin production and uptake (Shirk et al. 1992), and ecdysteroid profiles (Shaaya et al. 1993) during metamorphosis.

The oocytes of *P. interpunctella* contain two major yolk proteins: vitellin and follicular epithelium yolk protein (FEYP) (Shirk et al. 1984; Bean et al. 1988). Vitellin ($M_r = 475{,}000$), which is synthesized and secreted from the fat body as vitellogenin and accumulated in yolk spheres without major change, consists of subunits YP1 ($M_r = 153{,}000$) and YP3 ($M_r = 43{,}000$). The FEYP ($M_r = 235{,}000$) is produced by the follicular epithelial cells (FC) and consists of subunits YP2 ($M_r = 69{,}000$) and YP4 ($M_r = 33{,}000$).

The active process of vitellogenesis in pharate adult *P. interpunctella* begins during the fifth scotophase (96 h to 104 h after pupation; Zimowska et al. 1991; Shirk et al. 1992). Preceding the initiation of vitellogenesis, the ecdysteroid levels decline from the major pupal peak to below 500 pg/mg wet weight by 63 h after pupation (Shaaya et al. 1993), and the fat body completes the process of reassociation into the adult form by 80 h (Zimowska et al. 1991). Vitellogenin becomes detectable in the hemolymph at 83 h after pupation and steadily increases throughout the remainder of pharate adult development (Shirk et al. 1992). The terminal follicles, i.e. the follicles most posterior in ovarioles and closest to the calyxes, do not become vitellogenic until 96 h to 100 h after pupation (Zimowska et al. 1991). Although the initiation of vitellogenesis in *P. interpunctella* was shown to be dependent on the decline of the ecdysteroid titers (Shirk et al. 1990), the rate of growth for individual follicles and the initiation of specific steps in vitellogenesis appear to be regulated by additional factors (Zimowska et al. 1991; Shirk et al. 1992).

Using an immunoblot assay to detect the presence of the yolk protein subunits in whole ovary extracts, a temporal separation in the onset of subunit accumulation was observed (Shirk et al. 1992). The YP2 subunit of FEYP was first detected in ovaries at 96 h after pupation. However, the accumulation of vitellin was not detected in the ovaries until 116 h after pupation (Shirk et al. 1992). This temporal separation of YP2 and vitellin accumulation in whole ovaries has led us to question whether YP2 is produced and accumulated in the follicular epithelium prior to patency and then taken up by the oocyte with the other YPs or whether YP2 is taken up by the oocytes before the other YPs.

The approach taken in this investigation examines changes in the very first follicles that initiate vitellogenesis in *P. interpunctella*. To identify events during the initiation of vitellogenesis, we examined the subcellular distribution of the individual yolk protein subunits in terminal follicles. This study capitalizes on the synchronous development of pharate adults of *P. interpunctella* (Zimowska et al. 1991) and on our ability to detect the subunits of vitellin and FEYP with monospecific antisera for each of the subunits (Bean et al. 1988). From this work, we describe an extended transition stage of development, the provitellogenic stage, between the previtellogenic and vitellogenic stages of a follicle. We define the stages as follows: in the previtellogenic stage, the follicular epithelium is not patent, and the oocyte lacks yolk spheres. During the provitellogenic stage, the follicular epithelium is still not patent, but yolk spheres are present in the oocyte. However, during the provitellogenic stage, the yolk spheres contain qualitatively limited yolk protein. In the vitellogenic stage, the follicular epithelium is patent, and the oocyte contains yolk spheres with all yolk proteins present.

Materials and methods

Animal preparations. The *Plodia interpunctella* (Hübner) colony was reared according to Silhacek and Miller (1972) in a 16 h light:8 h dark cycle at 30° C and 70% relative humidity. Newly molted white pupae (±1 h) were collected at the beginning of a scotophase to obtain synchronous cohorts and then kept until the appropriate age. The predominant developmental stage at a specific time point was selected on the basis of external morphological characters as described by Zimowska et al. (1991) and accordingly used for tissue fixations.

Immunohistochemistry. The monospecific polyclonal rabbit antisera for YP1, YP2, YP3, and YP4 used in immuno-reactions were prepared as described in Bean et al. (1988). Preabsorbed antisera were prepared by serially exposing each of the antisera to the corresponding antigen electroblotted to nitrocellulose. The absence of antigen recognition by a preabsorbed antiserum was determined by exposing the blot to a goat-antirabbit-horseradish peroxidase conjugate (BioRad) and then developing a color reaction using an Immuno-Blot assay (BioRad). Background levels of immunoreactivity for each of the preabsorbed and nonreactive sera were determined by staining whole-mounts and paraffin sections of ovaries from 120 h pharate adults (data not shown). At this time, vitellogenic follicles were abundant and the reactivity of the sera could be tested against high levels of antigen.

Immunofluorescent detection of yolk polypeptides YP1, YP2, and YP3 in whole-mounts of ovaries was performed as described previously (Zimowska et al. 1991). The ovaries were incubated with one of the following as primary sera: one of the four monospecific rabbit YP antisera, one of the four preabsorbed monospecific YP antisera, or non-reactive serum diluted 1:250 in PBAT (0.1 M phosphate, pH 7.4, 1% azide, 1% Triton X-100). The secondary antiserum was a goat antirabbit IgG conjugated with fluorescein isothiocyanate (Sigma) diluted 1:40 in PBAT. Whole-mounts of ovaries in 80% glycerine diluted with 50 mM carbonate buffer (pH 9.4) were examined and photographed with an Olympus BHS microscope equipped with a BH2-RFL reflected light fluorescence attachment with a blue 490 nm excitation filter, a blue 455 nm supplementary exciter filter, and a G-520 barrier filter.

Immunogold-localization of the YPs in ultrathin sections was a modification of Leung et al. (1989). The ovaries were dissected in 0.8% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.5), 0.15 mM $CaCl_2$ and 4% sucrose and prefixed for 2 h at 4° C. The tissues were fixed overnight in 4% paraformaldehyde in phosphate buffer (pH 10.4) at 4° C. The tissues were dehydrated in a graded series of ethanol, and embedded in Lowicryl K4M (Chemische Werke Lowi). Ultrathin sections were collected on 200 mesh nickel grids coated with formvar (Ernest F. Fulam Inc.). The incubations were performed by floating a grid on a drop of medium and maintaining gentle agitation during each incubation at 24° C. The sections were etched with 3–6% H_2O_2 in double distilled H_2O for 5 min and then blocked with 3% BSA in TBS (0.5 m NaCl, 20 mM Tris, pH 7.5) for 25 min. The sections were incubated with one of the four monospecific YP antisera, one of the four preabsorbed monospecific YP antisera, or nonreactive serum diluted 1:250 in TBS plus 1% Tween-20 (TBS/Tween) for 60 min. After washing with TBS/Tween 3 times for 15 min with gentle agitation, the sections were exposed to goat anti-rabbit IgG linked with 20 nm colloidal gold (Polysciences, Inc.) diluted 1:5 in TBS/Tween for 60 min. After washing with 0.3% BSA in TBS, the sections were poststained with 2% uranyl acetate followed by 0.2% Reynolds' lead citrate (Reynolds 1963). Ultrastructural examination was performed on a Hitachi (H-600) transmission electron microscope operating at 75 kV.

Transmission electron microscopy

Ovaries were prefixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M sodium cacodylate, pH 7.5, 0.15 mM $CaCl_2$) at 4° C for 2 h and fixed in 1% OsO_4 in cacodylate buffer plus 2.5% sucrose at 4° C overnight. The tissues were dehydrated in a graded ethanol-acetone series and embedded in Epon-Araldite (Mollenhauer 1964). Ultrathin sections were poststained with 2% uranyl acetate followed by 0.2% Reynolds's lead citrate (Reynolds 1963). Ultrastructural examination was performed on a Hitachi (H-600) transmission electron microscope operating at 75 kV.

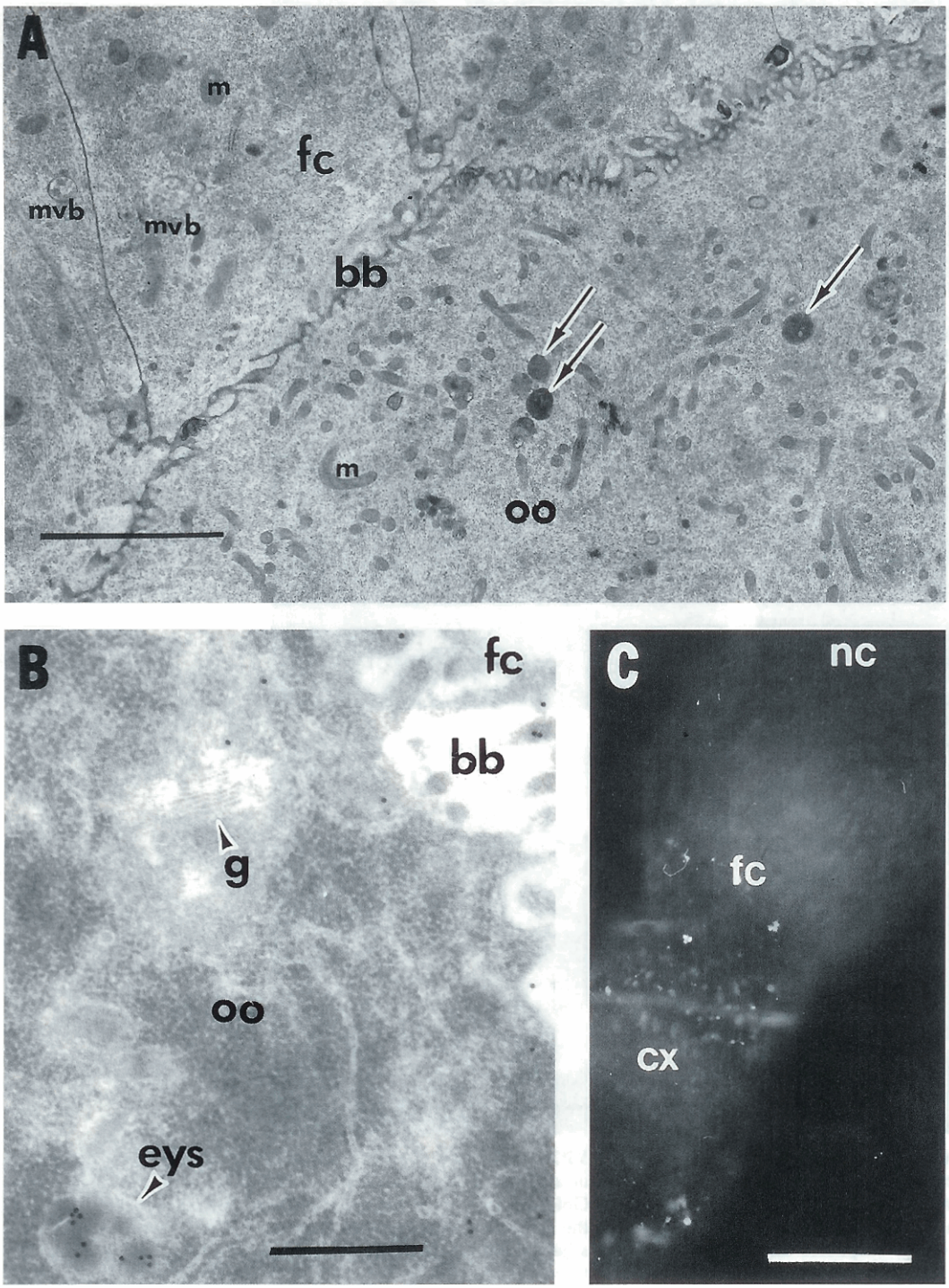

Fig. 1A–C. The localization of YP2 in conjunction with early yolk sphere formation in terminal follicles from 92 h pharate adult females. Panel (A) Electron dense early yolk spheres (*arrows*) near the brush border between the oocyte and follicular epithelial cells (TEM of epon section). Panel (B) Localization of immunogold labeled YP2 in early yolk spheres (TEM of Lowicryl section). Gold particles are 20 nm electron dense spheres. Panel (C) Immunofluorescent labeling of a whole-mounted ovary does not show fluorescence above background (compare labeling with 96 h terminal follicle in Fig. 4A). *bb*, brush border; *cx*, calyx; eys, early yolk spheres; *fc*, follicular epithelial cell; *g*, Golgi apparatus; *m*, mitochondrion; *mvb*, multivesicular body; *nc*, nurse cell cap; *oo*, oocyte. Magnification *bar* in panel (A)=2.5 μm, in pnael (B)=0.5 μm, and in panel (C)=25 μm

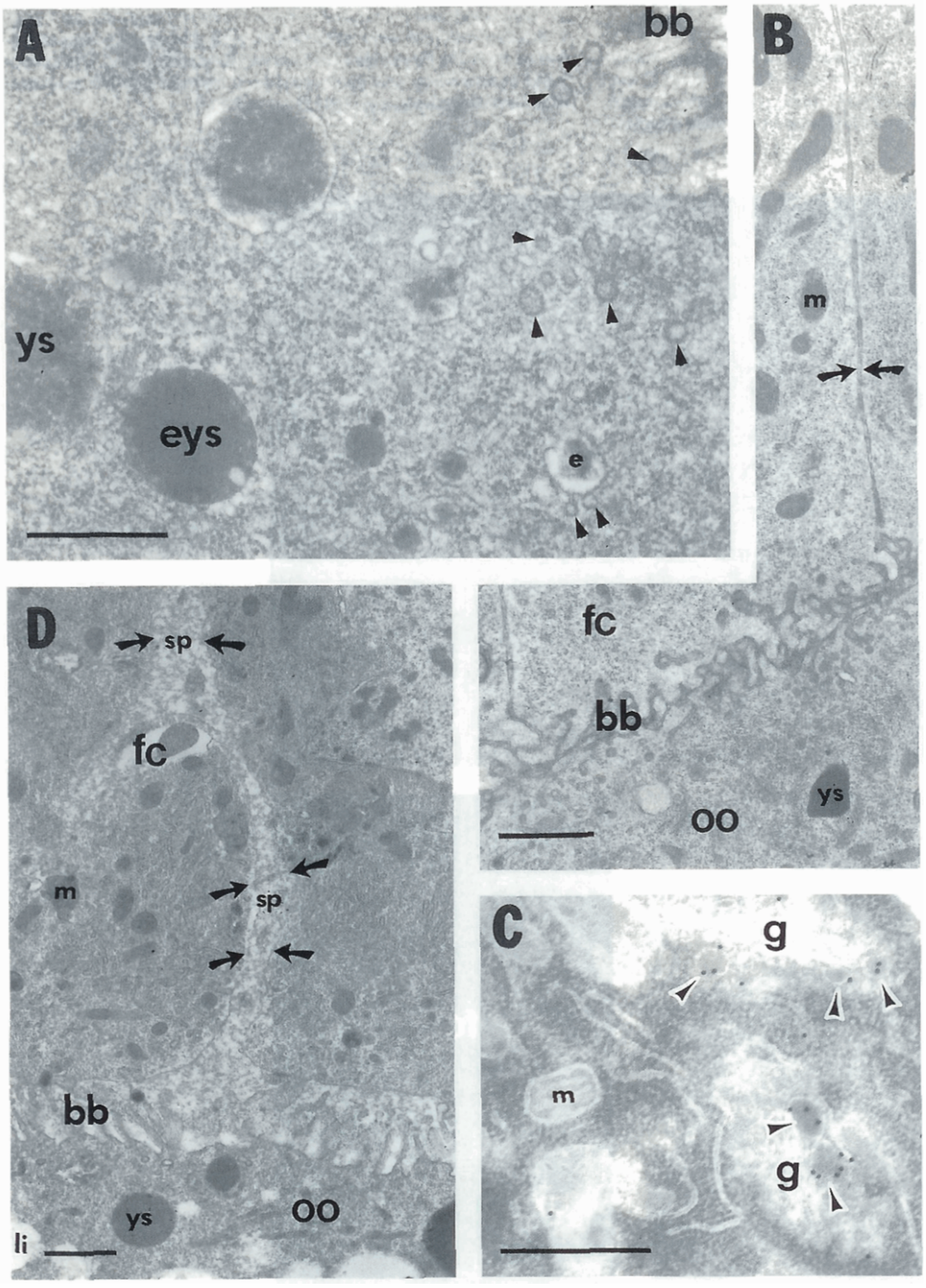

Fig. 2A–D. Yolk sphere formation occurs before the development of patency in terminal follicles of pharate adult females. **Panel (A)** The cytoplasm of a terminal oocyte from a 96 h pharate adult female contains numerous coated pits and vesicles (*arrowheads*), occasional endosomes, and early yolk spheres and yolk spheres near the brush border (TEM of Epon section). The early yolk spheres include small electron translucent vesicles that are absent from mature yolk spheres. Panel (B) At 105 h the follicular epithelium has small intercellular spaces between plasma membranes (between arrows) yet yolk spheres are present in the oocyte (TEM of Epon section). Panel (C) Localization of immunogold labeled YP2 in secretory granules (arrowheads) within Golgi complexes of a follicular epithelial cell at 105 h (TEM of Lowicryl section). Gold particles are 20 nm electron dense spheres. Panel (D) At 112 h the follicular epithelium has large intercellular spaces between the adjacent plasma membranes (between arrows) (TEM of Epon section). The intra-follicular cell matrix is continuous with the brush border. *bb*, brush border; eys, early yolk sphere; *fc*, follicular epithelial cell; *g*, Golgi apparatus; *li*, lipid droplet; *m*, mitochondrion; *oo*, oocyte; *sp*, inter-follicular cell space, ys, yolk sphere. Magnification bars in panels (A) and (D) = 0.5 μm and in panels (B) and (C) = 1.0 μm

Results

Activation of follicular epithelial cells and initiation of yolk uptake in terminal follicles

At 92 h after pupation, a few small (approximately 0.5 μm) yolk spheres were present and localized near the brush border of the oocytes in the terminal follicles of ovaries from pharate adult females (Fig. 1A). One class of yolk spheres, identified here as early yolk spheres, contained small electron translucent vesicles. In immunogold labeled ultrathin sections of these oocytes, the few early yolk spheres and yolk spheres that were present contained only YP2 (Fig. 1B). Neither YP4 nor vitellin (YP1 and YP3) were detectable in early yolk spheres (data not shown). Immunostaining of whole-mounted ovaries with antiserum to YP2 was not above background levels in either the oocyte or the FC (Fig. 1C).

In 96 h pharate adults which were at the beginning of the 5th scotophase, the terminal oocytes had begun to form numerous yolk spheres, while the follicular epithelium had small intercellular spaces (data not shown) and showed no signs of patency. In addition, the brush border of the terminal oocytes had increased numbers of microvilli with coated pits, and endosomes, early yolk spheres, and yolk spheres were observed close to the brush border of the oolemma but not whithin the central ooplasm (Fig. 2A). As was the case at 92 h after pupation, the early yolk spheres and yolk spheres within the terminal oocytes from 96 h pharate adults were immunogold labeled with antiserum to YP2 only (Fig. 3), while none of the other three antisera to YPs labeled the yolk spheres (data not shown).

Whole-mounted ovaries from 96 h pharate adult females contained sufficient amounts of YP2 in the terminal follicles so that the presence of YP2 in the cytoplasm of the FC and the oocytes was clearly discernable (Fig. 4). However, immunofluorescent staining of similar ovary preparations with antiserum to YP1 did not result in any significant fluorescence in either the oocyte or the inter-follicular cell spaces at this time (data not shown).

Development of patency and beginning of vitellogenin uptake in terminal follicles

The follicular epithelium of terminal follicles had initiated the development of patency by 105 h after pupation. The basal connections of the FC had begun to spread apart (data not shown) and immunofluorescent staining of whole-mounted ovaries showed limited reactivity for YP1 and YP3 in the expanding inter-FC spaces of the terminal follicle (Fig. 5C, D). However, strong reactivity for YP2 was observed in the FC of the terminal 10 follicles (Fig. 5A, B). Strong immunofluorescent reactivity for YP1 and YP3 was observed in the region of the inter-follicular connectives and, in particular, the spaces between the lumen cells (Fig. 5C, D). The presence of the immunoreactive material in this region may repre-

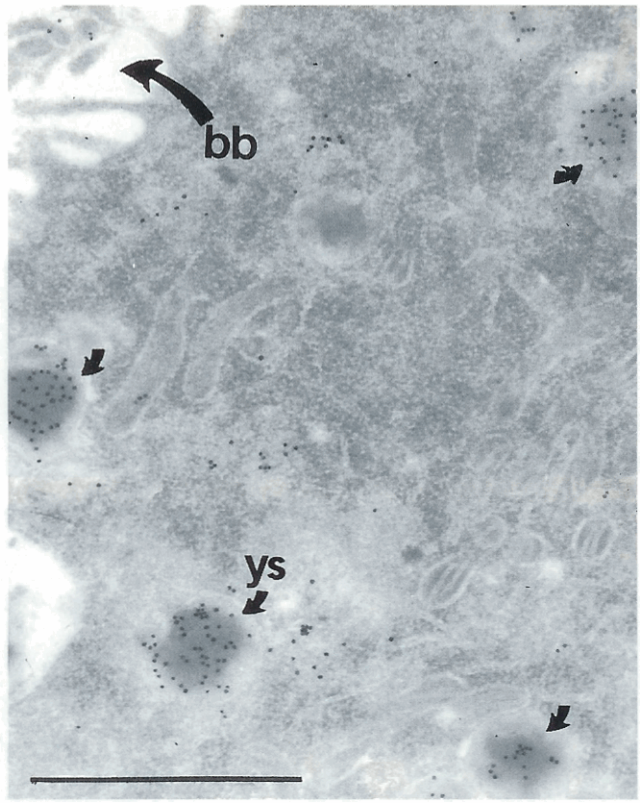

Fig. 3. At 96 h immunogold labeled YP2 is localized in yolk spheres of terminal oocytes from pharate adults (TEM of Lowicryl sections). *bb*, brush border; *ys*, yolk sphere. Magnification *bar* = 1 μm. The unlabeled *arrows* point to yolk spheres

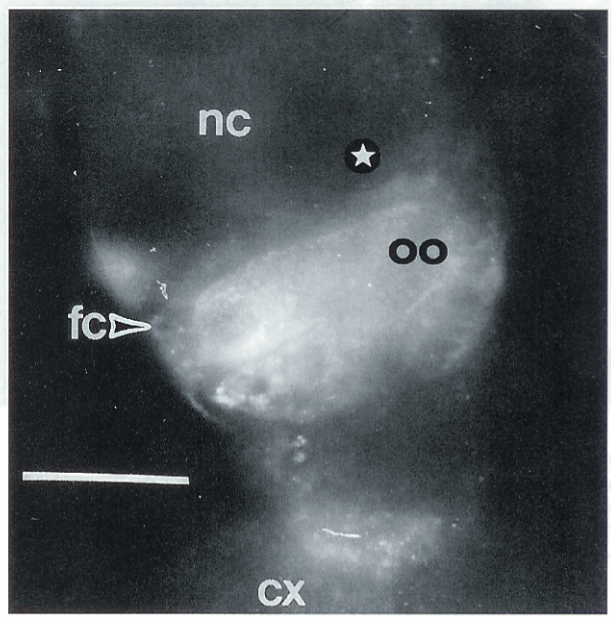

Fig. 4. The detection and localization of YP2 and vitellogenin (YP1) within terminal follicles of 96 h pharate adults, photomicrograph of whole-mounted follicle immunofluorescently stained for YP2. Immunofluorescently stained YP2 is detectable in the oocyte and its adjacent follicular epithelial cells but not in the nurse cells or their adjacent follicular epithelial cells. *cx*, calyx; *fc*, follicular epithelial cell; *nc*, nurse cell cap; *oo*, oocyte. Magnification *bar* = 25 μm. The star marks the terminal follicle in the ovariole

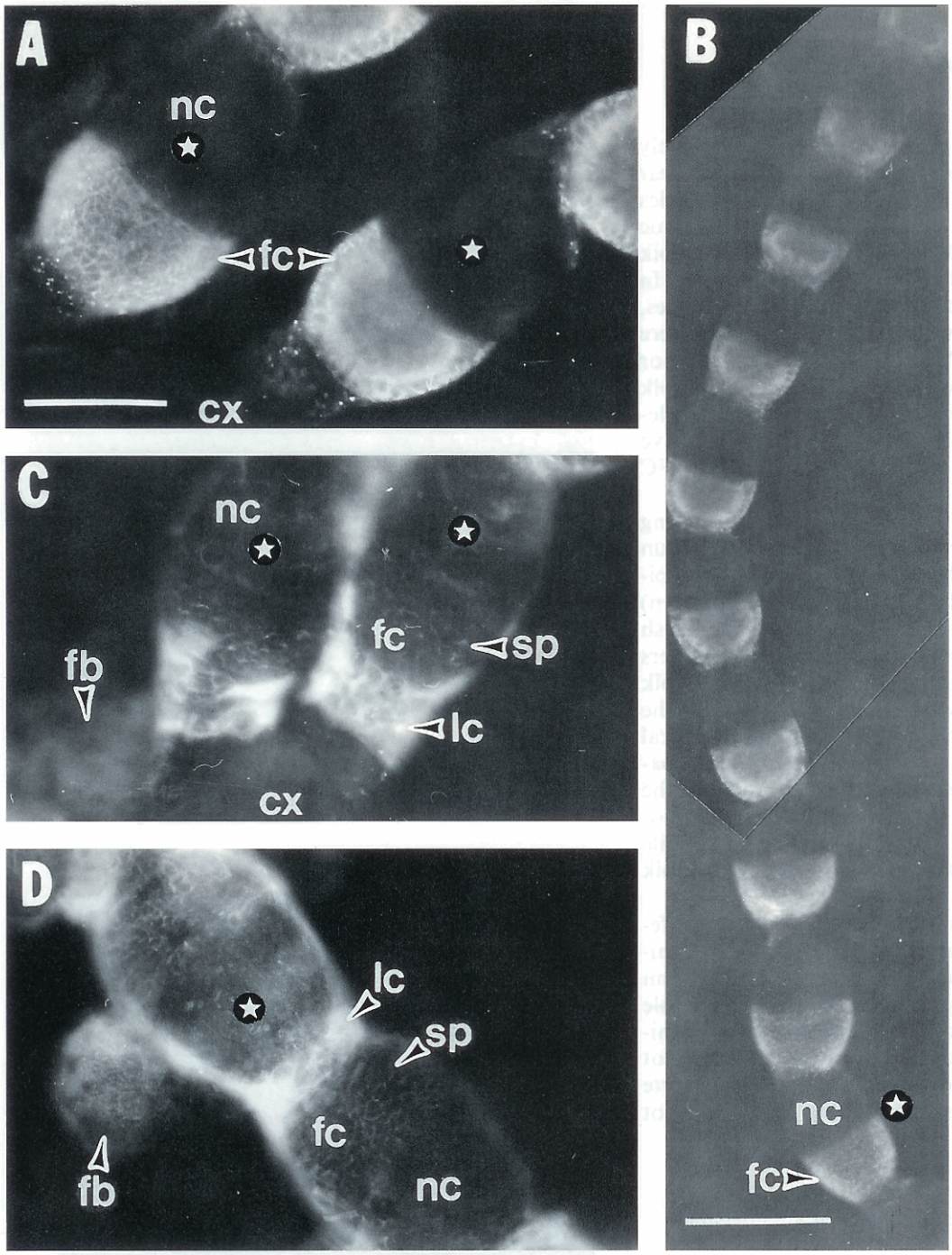

Fig. 5A–D. The detection and localization of YP2 and vitellogenin subunits (YP1 and YP3) within terminal follicles of 105 h pharate adults. The panels show photomicrographs of whole-mounted ovaries immunofluorescently stained for YP2 (**A** and **B**), YP1 (**C**), and YP3 (**D**). Immunofluorescently stained YP2 is present in the oocyte and its adjacent follicular epithelial cells (compare level of staining with 96 h, Fig. 4A, B). Immunofluorescently stained YP1 and YP3 were present in the area of the lumen cells and the interfollicular connective. Only traces of YP1 and YP3 were detected in inter-follicular cell spaces. *cx*, calyx; *fb*, fat body; *fc*, follicular epithelial cell; *lc*, lumen cells; *nc*, nurse cell cap; *sp*, interfollicular cell space. Magnification *bars* for (**A**, **C**), and (**D**) = 50 μm and the *bar* for panel (**B**) = 100 μm. The stars mark the terminal follicle in the ovariole

sent a pooling of vitellogenin (YP1/YP3) between the lumen cells. Although, YP1 and YP3 were detected in trace amounts in the inter-FC spaces, the spaces were small (Fig. 2B) and tight junctions were still evident between the FC (data not shown).

Immunogold labeling for YP2 in yolk spheres in terminal oocytes of 105 h pharate adult females was significantly greater than for YP1 and YP3 (Fig. 6A, C, D). The lower amounts of YP1 and YP3 (i.e. vitellin) in the yolk spheres of these females was most likely due

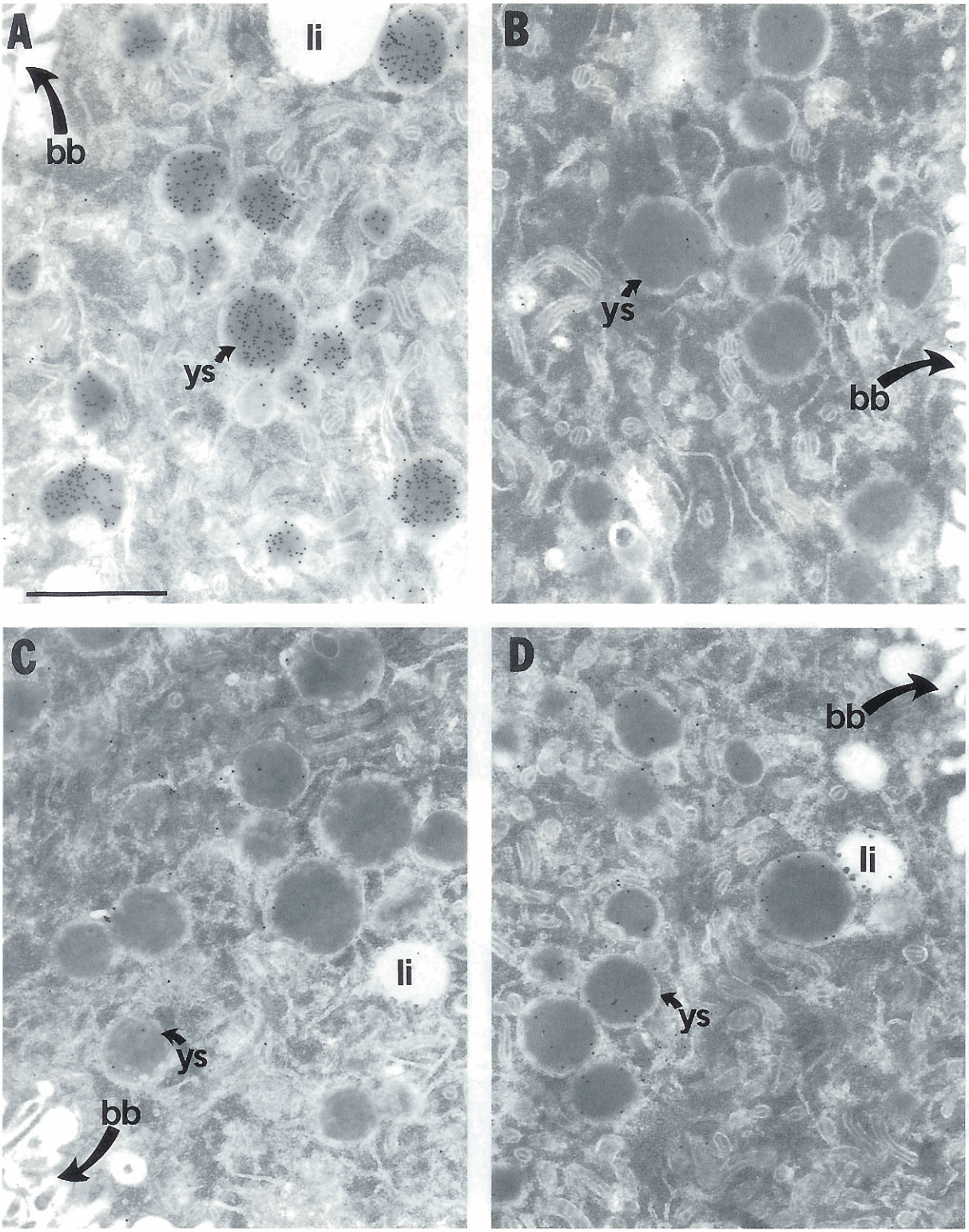

Fig. 6A–D. At 105 h immunogold labeled vitellin (YP1 and YP3) and YP4 were detectable at trace levels in yolk spheres of terminal oocytes from pharate adults. Immunogold labeling for YP2 (**A**) was much stronger than the minimal labeling for YP4 (**B**), YP1 (**C**), and YP3 (**D**) (TEMs of Lowicryl sections). *bb*, brush border; *li*, lipid droplet; *ys*, yolk sphere. Magnification *bar* = 1 μm

to the restricted availability of vitellogenin to the terminal oocytes because the follicular epithelium had not achieved patency.

The synthesis and secretion of YP2 by the FC became elevated as evidenced by the presence of YP2 in the forming secretory granules in the Golgi apparati (Fig. 2C) which, because of the scarcity of Golgi apparati, were not detected in earlier stages. No YP4 labeling

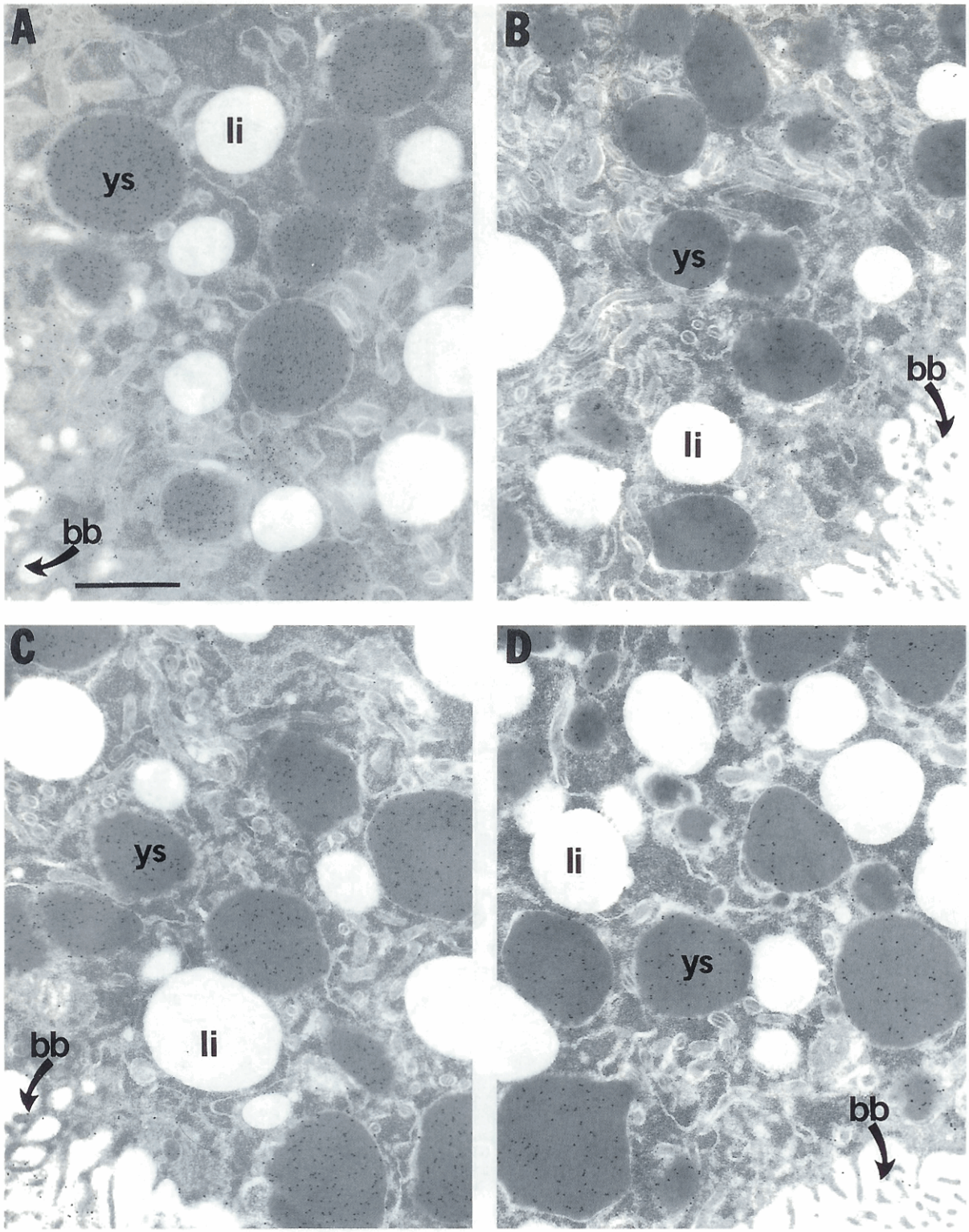

Fig. 7 A–D. At 112 h yolk spheres were equally immunogold labeled for the follicular epithelial cell protein subunits (YP2 and YP4) and the vitellin subunits (YP1 and YP3) in terminal oocytes from pharate adults. Immunogold labeling for YP2 (**A**), was equivalent to the labeling for YP4 (**B**), and the vitellin subunits, YP1 (**C**), and YP3 (**D**) in yolk spheres (TEMs of Lowicryl sections). *bb*, brush border; *li*, lipid droplet; *ys*, yolk sphere. Magnification *bar* = 1 μm

was observed in the secretory granules of the FC at this stage (data not shown) and only limited immunogold labeling of YP4 in the yolk spheres of the terminal oocytes (Fig. 6 B) was observed.

By 112 h after pupation, the terminal follicles were vitellogenic and the follicular epithelium had achieved patency. Ultrastructural examination of the follicular epithelium showed that large spaces existed between the

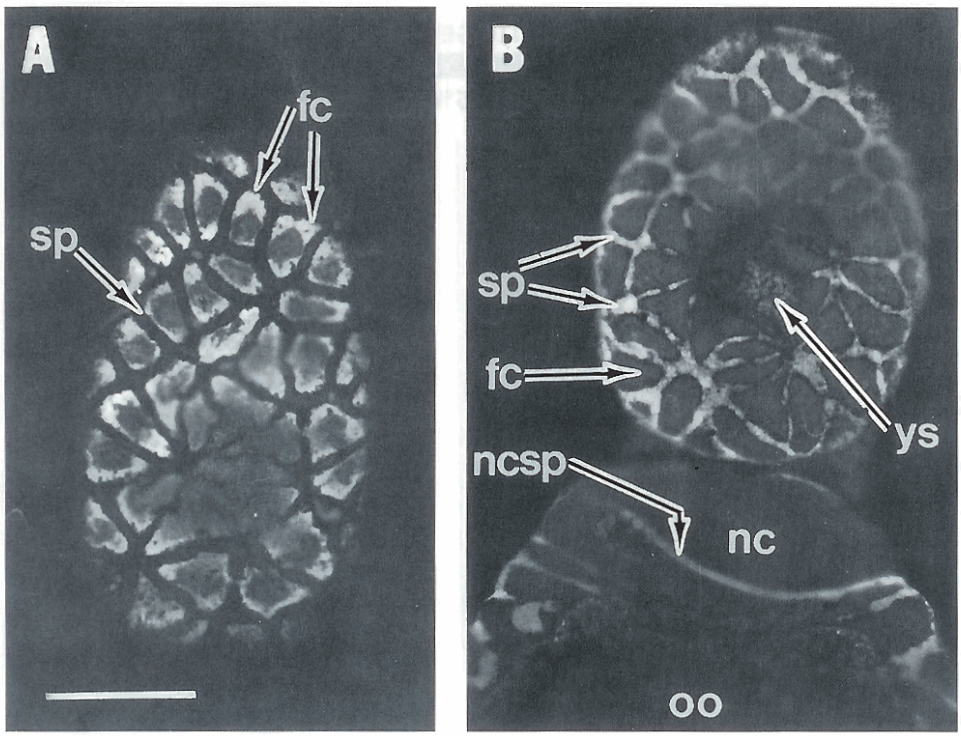

Fig. 8A, B. The detection and localization of YP2 and vitellogenin in vitellogenic terminal follicles of (120 h) pharate adults. The panels show immunofluorescent photomicrographs of semi-thick grazing sections. (**A**) Immunofluorescently stained YP2 was localized within the follicular epithelial cells. (**B**) Immunofluorescently stained vitellogenin (YP1) was observed in the interfollicular cell spaces. Immunofluorescently stained YP1 was observed also in the spaces between the nurse cells and in the yolk spheres within the oocyte. *fc*, follicular epithelial cell; *nc*, nurse cell cap; *ncsp*, inter-nurse cell space; *sp*, interfollicular cell space; *ys*, yolk sphere. Magnification for Panels (**A**), (**B**) = 25 μm

cells (Fig. 2D) and that few junctions remained between adjoining cells (data not shown). In addition, the oocytes contained numerous yolk spheres that were uniformly labeled by immunogold when reacted with antisera for each of the four YPs (Fig. 7). None of the yolk spheres, either near the brush border or within the ooplasm, labeled with only YP2 as had been observed in terminal oocytes from the earlier time points.

Fluorescent staining of whole-mounted ovaries from 120 h pharate adults showed strong reactivity for YP1 and YP3 in the inter-FC spaces of terminal follicles as well as strong reactivity for YP2 in the cytoplasm of the FC (data not shown). When these immunofluorescently stained whole-mounted ovaries were embedded in Lowicryl and sectioned, the semi-thick sections showed that the YP2 reactive material was restricted to the cytoplasm of the FC (Fig. 8A). The YP1 reactive material was observed in the inter-FC spaces as well as the spaces between the nurse-cells (Fig. 8B).

Discussion

Provitellogenesis: follicular transition from previtellogenesis to vitellogenesis and formation of YP2 yolk spheres

During the development of the terminal follicles of *P. interpunctella*, a transition period between the previtellogenic and vitellogenic stages was observed. The transition period or provitellogenic stage is marked in the beginning by the formation of yolk spheres containing YP2 and in the end by the development of patency by the follicular epithelium. The provitellogenic stage lasted approximately 13 h (extending from 92 h to 105 h after pupation; Fig. 9) which was half as long as the vitellogenic stage (approximately 30 h; from 105 h to 136 h). The provitellogenic stage thus represents a significant portion (10%) of the developmental time of a follicle.

The presence of immunogold labeled YP2 in vesicular organelles within terminal oocytes from 92 h old pharate adult females showed that the oocytes were actively accumulating a component of proteinaceous yolk and forming yolk spheres. Most likely, YP2 entered the oocyte by uptake across the brush border juxtaposed to the apical surface of the FC. This data is the first demonstration that the vesicles forming within the oocyte prior to the FC achieving patency are bona fide yolk spheres.

Electron dense vesicular organelles were observed also in stage 4 (non-patent) oocytes of Lepidoptera, such as *Anagasta kuehniella* (Cruickshank 1971, 1972), *B. mori* (Yamauchi and Yoshitake 1984), and *H. cecropia* (King and Aggarwal 1965), and in late non-patent, previtellogenic (stage 7) oocytes of *Drosophila melanogaster* (Cummings and King 1970; Mahowald 1972; Giorgi and Jacob 1977). These investigators concluded that the electron dense vesicular organelles contained yolk on the basis of morphological criteria, although there was no immunohistochemical confirmation of the identity of yolk proteins within the vesicles. To account for the presence of yolk spheres in previtellogenic oocytes of *D. melanogaster*, Cummings and King (1970) and Mahowald (1972) suggested that the yolk was synthesized within the oocyte. On the other hand, Giorgi and Jacob (1977) suggested that these organelles were the result of autophagic activities of the oocyte. Because the FC of moths and flies produce yolk proteins (Bast and Telfer 1976; Brennan et al. 1982; Issac and Bownes 1982; Irie

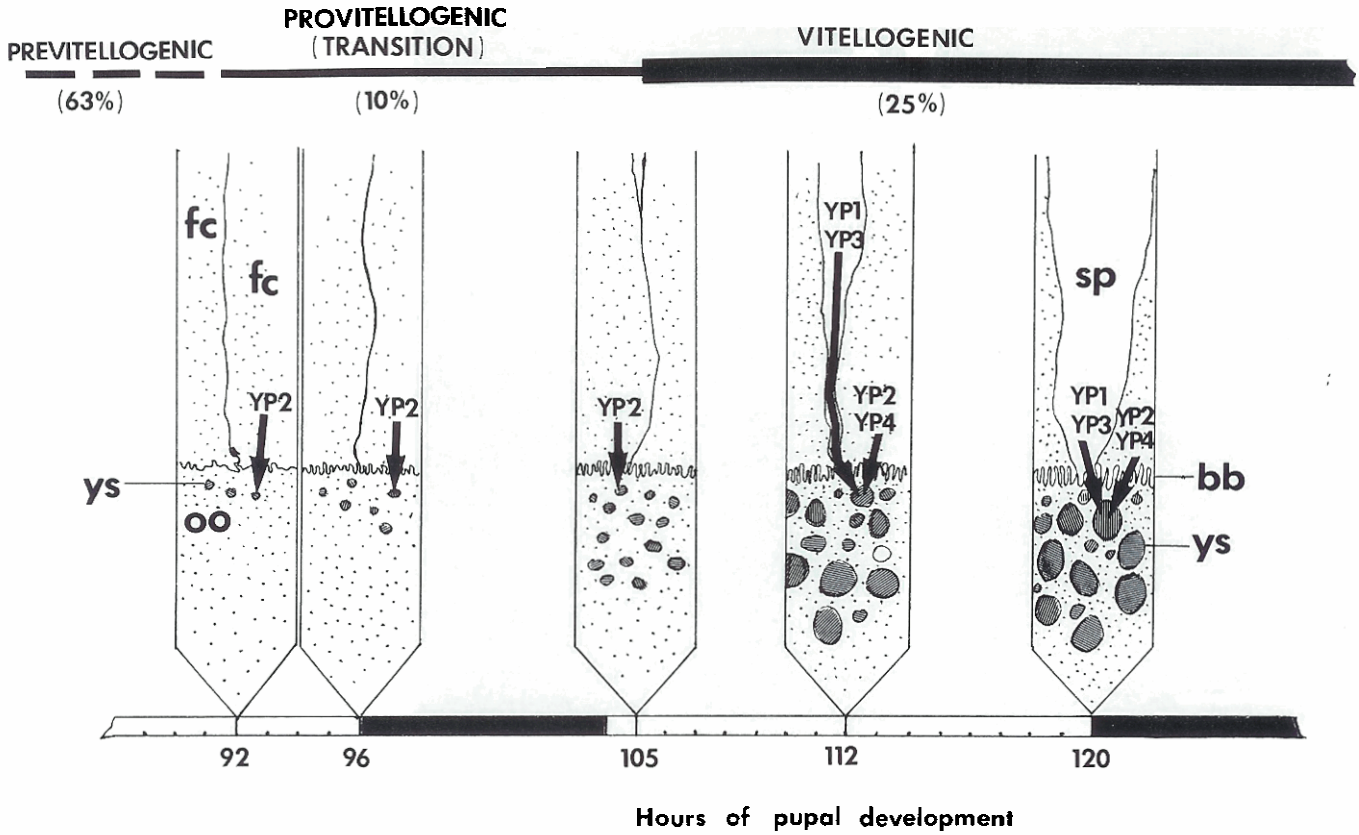

Fig. 9. The temporal sequence for yolk sphere formation during the transition of terminal follicles from previtellogenic to vitellogenic activities in ovaries of pharate adult female *Aedes aegypti*. The *arrows* show the origin and route of entry for the yolk proteins found in the yolk spheres of the oocyte at various times during the provitellogenic and vitellogenic stages. The percentages under each stage represent the proportion of time that each stage lasts of the total time of follicle development (138 h: Zimowska et al. 1991). The previtellogenic period encompasses both the time of follicular formation and the organized follicle. The black bars on the time scale represent the scotophases. *bb*, brush border; *fc*, follicular epithelial cells; *oo*, oocyte; *sp*, interfollicular epithelial cell space; *YP*, yolk polypeptides; *ys*, yolk spheres

and Yamashita 1983; Shirk et al. 1984; Shirk 1987), the yolk present in yolk spheres of the non-patent oocytes from these insects most likely was produced in the follicular epithelium, transferred to the oocyte across the brush border, and deposited in yolk spheres in a process similar to that described in this investigation. Transfer of yolk proteins from the FC through the oolemma to the oocyte was also observed in vitellogenic follicles of *D. melanogaster* (Butterworth et al. 1992).

During the provitellogenic stage after yolk sphere formation was initiated, there was a rapid proliferation of the yolk spheres in the oocyte. YP2 was the only proteinaceous yolk detectable in the yolk spheres during the provitellogenic stage, and once formed, the size of the individual yolk spheres remained small until the vitellogenic stage (Zimowska et al., unpublished work). These observations lead us to examine the possibility that the formation of yolk spheres may be dependent on the presence of YP2.

The proliferation of YP2 yolk spheres during the provitellogenic stage also suggests that the oocyte is assembling organelles necessary for the rapid uptake of yolk after the follicles achieve patency. This is reminiscent of the juvenile hormone stimulated period of previtellogenic activity in *A. aegypti* (Raikhel and Lea 1985) where there is a development of a specialized cortex around the oocyte to support the subsequent yolk uptake. Although there was an increased convolution associated with the oolemma of the moth, there was no apparent accumulation of endosomes and coated pits near the brush border of the oocytes. This difference may be due to the inability of the mosquito to form yolk spheres because of the exclusion of the yolk proteins from the follicle until a later time while the moth does not accumulate these organelles because yolk protein is available from the follicular epithelium and yolk sphere formation proceeds. Even though there may be differences in the mechanisms between the moth and the mosquito, the stages may serve the same functional activity of providing the oocyte with the necessary cellular machinery to accomplish yolk formation as quickly as possible following initiation of vitellogenesis.

Patency, yolk formation, and vitellogenesis

YP1 and YP3 (subunits of vitellin) were not detected in the inter-follicular spaces and yolk spheres of terminal

follicles from *P. interpunctella* until after the spaces between the FC began to develop. At 105 h after pupation when the inter-FC spaces were beginning to open, traces of the other three YPs were observed in the yolk spheres but at levels much lower than that of YP2. This point in development provided the first evidence that the follicular epithelium of the terminal follicles was beginning to develop patency. Equivalent levels of labeling for the subunits of vitellin and FEYP were not detected in the yolk spheres until 112 h after pupation and this was coincident with the follicular epithelium achieving patency (Fig. 9). The observations from this investigation support our previous report of the separated temporal appearance of the YPs in ovaries of late pharate adult females which was based on detection of YPs in ovarian extracts by Western blotting (Shirk et al. 1992). However, the data presented here more accurately establish the timing of the these events in the individual terminal follicles within the ovaries.

The recognition of an extended transition period between the previtellogenic stage, where no yolk sphere formation is occurring, and the vitellogenic stage, where rapid yolk sphere formation occurs, brings into question when the vitellogenic stage actually begins. In *H. cecropia*, a follicle has been considered to be vitellogenic when it has developed patency and begun to rapidly accumulate extra-ovarian protein (Telfer et al. 1982). A functional approach to this question for *P. interpunctella*, therefore, would place the beginning of the vitellogenic stage in terminal follicles between 105 h and 112 h when patency develops and vitellin appears in the yolk spheres. Consequently, the period between 92 h and 105 h would not be considered vitellogenic even though yolk spheres are present.

After the follicular epithelium achieved patency, the size of the yolk spheres increased rapidly, and the number of yolk spheres continued to increase (Zimowska et al., unpublished work). In addition, the yolk proteins became evenly distributed throughout all of the yolk spheres as evidenced by uniform labeling of the yolk spheres for all four YPs. Even though the yolk spheres formed before 112 h contained only YP2, no yolk spheres containing only YP2 were observed after 112 h. These data indicate that as the yolk spheres mature, the smaller spheres fuse and the yolk mixes resulting in a homogeneous proteinaceous yolk within the mature yolk spheres.

As was the case for vitellin, the FEYP subunit YP4 was not detected in yolk spheres within the terminal oocytes until 112 h after pupation. This observation shows that the appearance of YP2 and YP4 in follicles is not coordinately regulated regardless of their being subunits of a major yolk protein (Shirk et al. 1984; Bean et al. 1987) and their simultaneous presence in secretory granules of FC at later stages of follicular development (Zimowska et al., unpublished work). Although YP4 was not detected in the yolk spheres of terminal oocytes until 112 h, trace amounts of YP4 were detected by immunofluorescence in FC of previtellogenic follicles as early as 33 h after pupation (Zimowska et al., unpublished work).

Further evidence that YP2 and YP4 do not share coordinate regulation has been found when follicles terminate vitellogenesis (Zimowska et al., unpublished work). Beginning with the transition from the vitellogenic stage to vitellin membrane synthesis stage, YP2 was no longer detectable in the follicular epithelium, while the presence of YP4 continued even into the choriogenic stage. Therefore, a more complete understanding of the temporal regulation of these two YPs requires quantitative analysis of the transcript levels and correlation with protein levels for each YP during this developmental period.

From this study, the increase in YP4 levels in the terminal follicles was shown to be concurrent with the development of patency by the follicular epithelium. The concomitant development of patency with increased production of YP4 implies that a common mechanism(s) of activation may operate for each event.

Regulation of follicle development during the transition from previtellogenic to vitellogenic stage

The initiation of active yolk uptake and the formation of yolk spheres by the oocyte prior to the initiation of patency by the follicular epithelium suggests a stepwise mechanism of activation of the vitellogenic processes. These periods of rapidly changing activity state in follicles indicate that there may be regulators, originating either extra- or intrafollicularly, that influence the metabolic activities of the tissues within each follicle.

A rapid change in the activity state and the ionic coupling between nurse cells and oocyte was observed during initiation of vitellogenesis in follicles of *H. cecropia* (Woodruff and Telfer 1990). The changes in activity state and ionic coupling were thought to be the consequence of a single control cascade (Woodruff and Telfer 1990). From this study, the initiation of vitellogenesis in terminal follicles from *P. interpunctella* appears to occur in a sequence with at least two steps and implies the existence of a regulatory cascade as well. However, the regulatory mechanisms and the temporal organization of the initiation of vitellogenesis in follicles, including ionic coupling, as observed in *H. cecropia*, along with yolk sphere formation, yolk uptake and patency, as observed in *P. interpunctella*, have not been established. Because we are able to examine changes in the terminal follicles as the process of vitellogenesis begins in the ovaries of pharate adult female *P. interpunctella*, further analysis of this process should provide a model to determine the nature and source of regulatory mechanisms.

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