1.3 Vitellogenesis and Post-Vitellogenic Maturation of the Insect Ovarian Follicle

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1.3.1. Introduction

Female insects typically produce prodigious numbers of eggs to assure the propagation of their genes, and invest considerable resources towards this end. Ultimately, the egg of an insect must contain a haploid set of chromosomes, sufficient nutrients to supply the growing embryo with resources to last until the larva or nymph ecloses and begins feeding, and a set of determinants to direct the organization and progression of embryogenesis, including the differentiation of a new cluster of germ cells. As with all organs, the morphology of the ovary reflects the physical and genetic requirements of its physiological role, which in this case is the functional assembly of the various components of the oocyte.

Visual inspection shows that the polytrophic ovary of holometabolus insects, which represent a major focus of this chapter, is comprised of a series of ovarioles that contain linear arrays of progressively developing follicles starting with dividing germ stem cells at one end and ending with mature oocytes ready for fertilization at the other (**Figure 1**). Essentially, the ovariole can be considered an assembly line leading to the production of the egg. How this assembly line operates within different species to produce similar end products, i.e., the mature oocytes, depends on the insect, its life style, and its evolutionary history.

1.3.2. Previtellogenesis: The Development of the Ovarian Structure

In this section, the development of the ovary and oocyte in the fruit fly, *Drosophila melanogaster*, is examined briefly and then extend the discussion to other insects, mainly Lepidoptera and non-*Drosophila* Diptera, where most of the additional relevant information is available.

1.3.2.1. *Drosophila melanogaster*

The organogenesis of the ovary and the production of eggs in *Drosophila* is one of the most completely examined developmental systems. From the formation of germ cells in the embryo to the localization

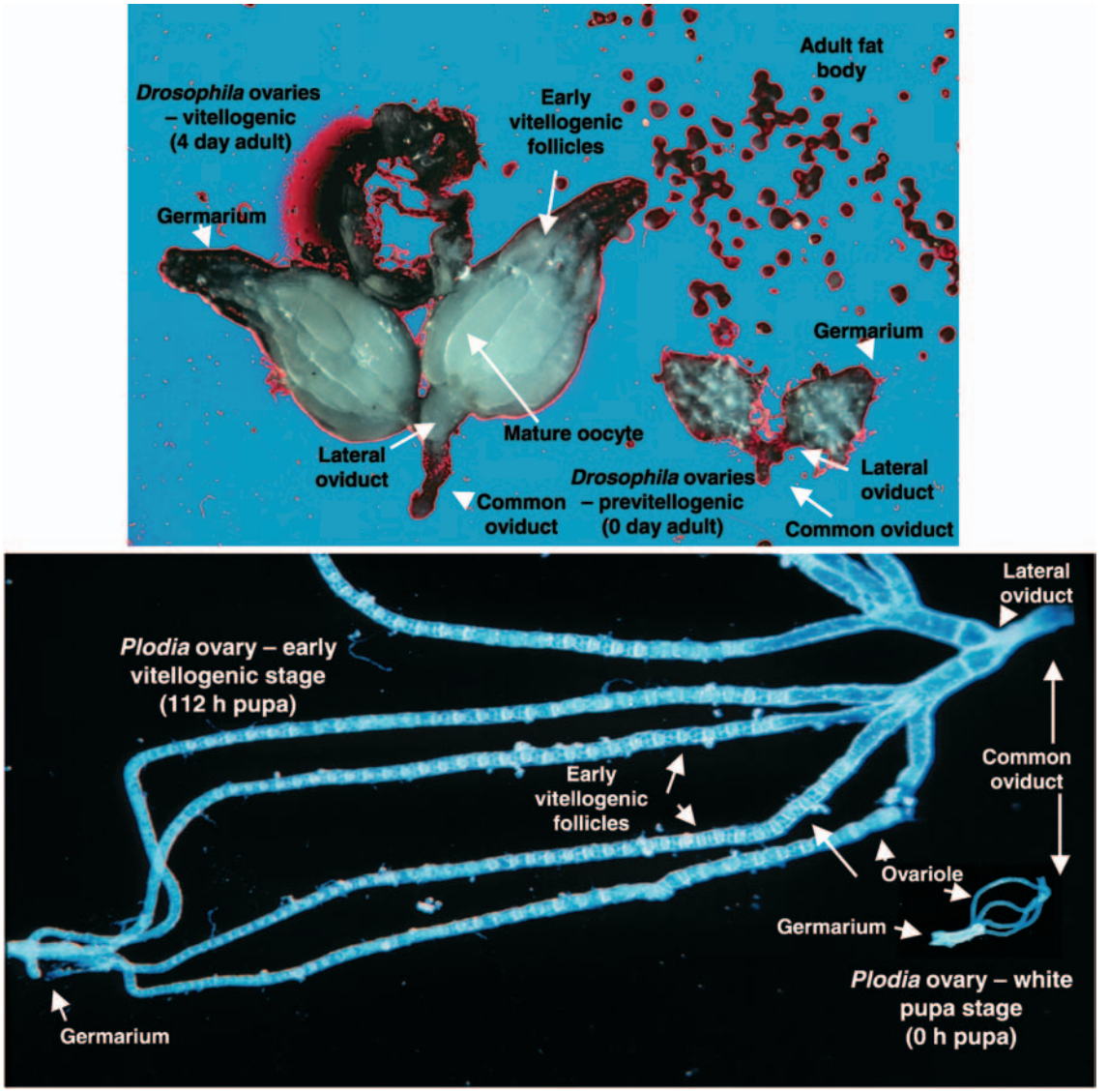


Figure 1 Ovaries of holometaboulous insects. The upper panel shows ovaries dissected from previtellogenic (newly eclosed) and vitellogenic (four day old) *Drosophila melanogaster* adult females. The increase in size of the ovaries is due to the uptake of yolk proteins and other nutrients by the maturing follicles. The lower panel shows ovaries dissected from early vitellogenic (112 h) and prefollicular (white or 0 h) *Plodia interpunctella* female pupae. The increase in size is due to the growth of the germ and somatic tissues of the ovary. The linear array of developing follicles can be seen within each ovariole extending from the germarium to the lateral oviduct. The size of the ovary will increase again as the follicles complete vitellogenesis.

of germ cell determinants in the oocytes within the ovary of the adult female, the growth and activities of the ovary and follicles have been examined extensively to determine the fundamental genetic and developmental processes that are involved in the production of an egg. As a result, the structural organization of the ovary and the developmental processes controlling its formation have been studied in-depth for over three-quarters of a century (Dobzhansky, 1930; Kerkis, 1930; Demerec, 1950; King *et al*., 1968; King, 1970; Mahowald and Kambysellis, 1980; Spradling, 1993; Lasko, 1994; Mahajan-Niklos and Cooley, 1994; Dobens and Raftery, 2000; Houston and King, 2000) (see **Chapter 1.2**).

In *Drosophila*, the female molts into the adult stage with an immature ovary but, under the influence of juvenile hormone (JH), it initiates yolk protein production (vitellogenesis) and follicle maturation within the first day of adulthood (reviews: Postlethwait and Shirk, 1981; Bownes, 1986; Lasko, 1994). The female continues producing eggs throughout its life, provided a sufficient supply of nutrients is available (Bownes, 1986). Because the process of egg production is continuous, plentiful experimental material is always at hand, providing

a rich resource for the manipulation and analysis of oocyte production and maturation.

Under the influence of determinants localized within the posterior pole plasm of the embryo, the germ cells are the first to cellularize in the syncytial embryos (reviews: Spradling, 1993; Houston and King, 2000) (see **Chapter 1.2**). The formation of the pole plasm within the oocyte is dependent upon the maternal contribution and posterior localization of transcripts for *oskar* (*osk*; Lehman and Nüsslein-Volhard, 1986), *nanos* (*nos*; Nüsslein-Volhard *et al*., 1987), and *germ cell-less* (*gcl*; Jongens *et al*., 1992, 1994), as well as the proteins Staufen (Stau; Schüpbach and Wieschaus, 1986), Valois (Vls; Schüpbach and Wieschaus, 1986), Vasa (Vas; Schüpbach and Wieschaus, 1986), Tudor (Tud; Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986), and Mago-nashi (Mago; Boswell *et al*., 1991). In addition to these determinants, normal germ cell development and dorsoventral patterning are also dependent on the posterior localization of Cappuccino (Capu) and Spire (Spir) (Manseau and Schüpbach, 1989) through the organization of microtubules and regulation of cytoplasmic streaming. Furthermore, germ cell development and posterior somatic patterning require posterior localization of the *nos* and *pumilio* (*pum*) transcripts (Lehman and Nüsslein-Volhard, 1987). These transcripts and proteins are produced in the nurse cells and sequentially localized in the posterior of the embryo. The transcripts include a 630-nucleotide sequence within the 3′ untranslated region, which specifies the localization signal that interacts with microtubules to facilitate translocation (Macdonald and Struhl, 1988; Pokrywka and Stephenson, 1991, 1995; review: **Chapter 1.2**). Once associated with the microtubules, the translocation of these determinants from the nurse cells to the oocyte and their stable localization within the embryo is dependent on activities of the microtubules, which function both via minus- and plus-end-directed motors driven by dynein and kinesin, respectively (reviews: Pokrywka, 1995; Stebbings *et al*., 1995) (see **Chapter 1.2**).

The number of germ cells formed in the embryo is influenced by the levels of Osk (Smith *et al*., 1992). A threshold level of Osk is required for normal germ cell formation and establishment of normal body patterning, while its over-expression leads to ectopic formation of pole cells as well as formation of additional posterior pole cells (for additional details, see **Chapter 1.2**). After cellularization of the germ cells, their migration and eventual localization within the gonads can be visualized using antibody staining for the Vas protein (Lasko and Ashburner, 1990), an RNA helicase that is constitutively expressed in germ cells (Hay *et al*., 1988; Lasko and Ashburner, 1988). Nearly half of the originally formed pole cells are lost during embryogenesis, as they migrate from the posterior of the germ band to their position within the embryonic gonads, and only 10–15 germ cells are present within the late embryonic gonad (Lasko and Ashburner, 1990; Mueller, 2002) (see **Chapter 1.2**).

Throughout most of the larval stages, the germ cells divide mitotically to produce an unorganized mass of primordial germ cells mixed with somatic cells (Kerkis, 1930). Midway through the third instar, however, the metamorphosis of the ovary is initiated, leading to the organization of the somatic and germ cells into the adult organ (King *et al*., 1968) (see **Chapter 1.2**). The morphogenetic changes in the undifferentiated ovary begin approximately 12 h after the molt to the third (last) instar, when the anterior somatic cells of the ovary express Ultraspiracle (USP; Oro *et al*., 1990) and the A isoform of the ecdysteroid (or ecdysone) receptor (EcR-A; Hodin and Riddiford, 1998). In the presence of low levels of ecdysteroids, these two proteins interact to activate downstream gene expression including that of the *bric à brac* (*bab*) gene. Under the influence of the BAB protein, approximately 20 terminal filament stacks begin to differentiate from the somatic cells (Godt and Laski, 1995; Sahut-Barnola *et al*., 1995).

After initiating differentiation, the terminal filament cells maintain high levels of USP but no detectable EcR (Hodin and Riddiford, 1998). The first two terminal filament stacks form on the medial side of the ovary, with new stacks forming more laterally, as progressively more are added until 32 h after the molt (Godt and Laski, 1995). By the time of pupariation, the 13–20 terminal filaments, which consist of stacks of 8–9 disk-shaped cells, have been formed in the ovary. These terminal filament stacks establish the positions for the formation of each of the ovarioles within the ovary.

Within 48 h from pupariation, the morphogenesis of the ovary is completed. A small cluster of cells forms at the base of the terminal filaments, in direct contact with the 2–3 germ cells localized within each ovariole (Lin, 2002) (see **Chapter 1.2**). The somatic cap cells and inner sheath cells surround the germ cells and form a niche that provides the signaling that controls germ cell divisions during the adult reproductive period (see **Chapter 1.2**). The primary germ cells function as stem cells and divide asymmetrically to produce one daughter cell that becomes a cystoblast, which enters follicle differentiation, and another daughter cell that retains a stem cell function (reviews: Spradling *et al*., 2001;

Lin, 2002) (see **Chapter 1.2**). The cap cells, alternatively, express several genes, e.g., *hedgehog* (*hh*), *piwi*, *fs(1)Yb*, and *decapentapalegic* (*dpp*), whose function is required for the maintenance of stem cell function (Cox *et al*., 1998; Xie and Spradling, 1998; King and Lin, 1999; Cox *et al*., 2000; Xie and Spradling, 2000; King *et al*., 2001) (see **Chapter 1.2**). Of these genes, *dpp* is the major regulator for clonal expansion of germ cells during the formation of the niche at the early pupal stage (Zhu and Xie, 2003).

The establishment of the stem cells within the niche requires fewer germ cells than are present at pupation, and stem cells that are not included in the niche differentiate directly into cystoblasts (Bhat and Schedl, 1997). Establishment of the germ stem cells is also dependent on the formation of *adherens* junctions between the cap cells and germ stem cells (Song *et al*., 2002). In addition to establishing their identity, they also provide a further physical constraint leading to asymmetric division of the germ stem cells, which is important for the renewal of the stem cells (Zhu and Xie, 2003).

Each ovariole in a mature ovary is divided into three regions, the terminal filament, the germarium, and the vitellarium (**Figures 1** and **2**). Within the germarium, four functional regions, 1, 2A, 2B, and 3, have been described (Koch and King, 1966; Mahowald and Strassheim, 1970; Lasko, 1994) (see **Chapter 1.2**), where the previtellogenic follicle forms (**Figure 2**). A cluster of 2–3 germ stem cells is found within a niche in the anterior-most section of the germarium (region 1), immediately adjacent to the terminal filaments (Wieschaus and Szabad, 1979). During egg production by the adult female, germ stem cells divide asymmetrically to produce cystoblasts and new stem cells (review: Lin, 2002; **Chapter 1.2**). The asymmetry of the cell division is necessary for maintenance of the stem cells, so that eggs can be produced throughout the adult stage.

In addition to anchoring the germ stem cells to the cap cells, polarized division of the daughter cells is maintained by the asymmetric segregation of the fusome (or spectrosome) in the germ stem cell. The fusome is a ring-shaped organelle replete with membrane skeletal proteins including actin (Warn *et al*., 1985; Theurkauf *et al*., 1992), α- and β-spectrins (de Cuevas *et al*., 1996), filamin and ankyrin (Li *et al*., 1999), motor molecules such as cytoplasmic dynein (McGrail and Hays, 1997), and the unique fusome proteins Hu-Li Tai Shao (HTS, an adducin-like protein; Yue and Spradling, 1992; Ding *et al*., 1993), Bag-of-marbles (BAM; McKearin and Ohlstein, 1995), TER94 (León and McKearin, 1999), and Kelch (Xue and Cooley, 1993). The fusome acts in

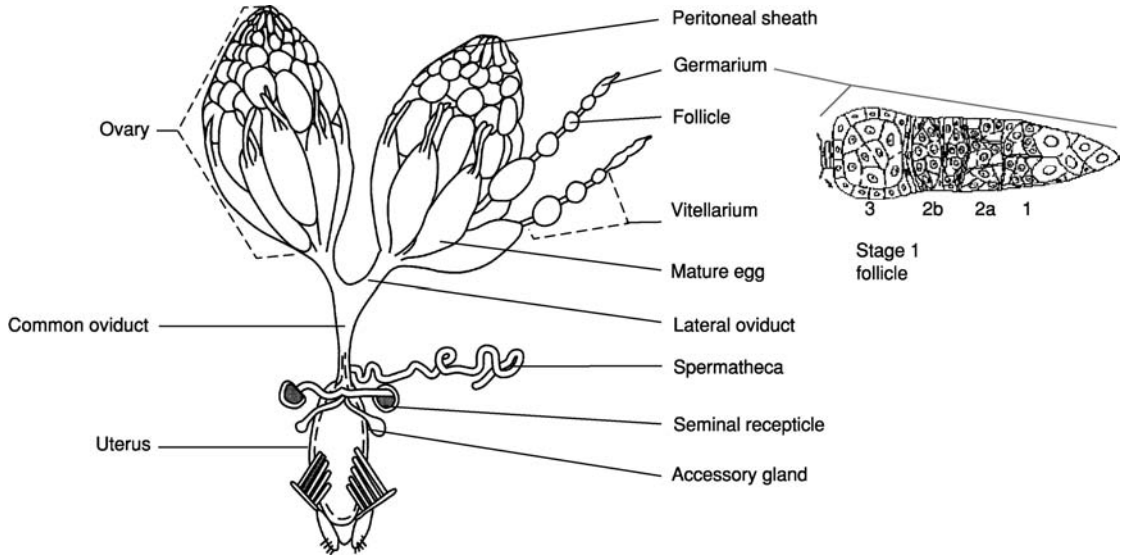


Figure 2 The reproductive system of *Drosophila melanogaster* contains two ovariole clusters that connect to a common oviduct. Eggs pass through the oviduct and are momentarily stored in the uterus until egg deposition. The broken line indicates the position of the mature egg in the uterus (the posterior end faces down). (Redrawn from Miller, A., **1950**. The internal anatomy and histology of the imago of *Drosophila melanogaster*. In: M. Demerec, M. (Ed.), Biology of *Drosophila*. Wiley, New York, pp. 420–534). The germarium contains germline stem cells (region 1) and mitotically active 2-, 4-, and 8-cell clusters. Clusters of 16 germline cells are present in 2a, where follicle cells begin to migrate around the cluster. Young egg chambers completely surrounded by follicle cells flatten into a lens shape in region 2b. The egg chamber reaches region 3 of the germarium (stage 1), where it becomes spherical and prepares for departure from the germarium as a stage 2 egg chamber. (Redrawn in part from Robinson, D.N., Cant, K., Cooley, L., **1994**. Morphogenesis of *Drosophila* ovarian ring canals. *Devel. 120*, 2015–2025).

determining the asymmetrical division of the dividing germ stem cells by associating with the cap cell-anchored germ stem cell pole of the mitotic spindle in subsequent mitoses of the cystocytes (Storto and King, 1989; Lin and Spradling, 1995; McGrail and Hays, 1997; Máthé *et al*., 2003) (see **Chapter 1.2**).

The polarization of fusome localization is accomplished under the influence of *pum* whose product is involved in the organization of the microtubule structure (Lin and Spradling, 1997), and the Orbit/Mast and CLIP-190 proteins, which mediate interactions between microtubules and cytoskeletal organelles (Máthé *et al*., 2003). The Orbit/Mast protein is found initially at the poles of the mitotic spindle but subsequently moves, coincident with the arrested cleavage furrow. This guides the migration of the fusome plugs toward the site of the pre-existing fusome (Storto and King, 1989), and assures the polarized positioning of the fusome and the asymmetry of germ stem cell division.

Following its separation from the germ stem cell, a cystoblast divides four times, with incomplete cytokinesis, within region 2A. This produces the cystocyte complex. The complex is composed of the oocyte that is connected by cytoplasmic bridges to the 15 nurse cells. The asymmetric division of the cystocytes leads to the assignment of one cell as the oocyte, with the remaining daughters of the cystocyte progeny becoming nurse cells (Storto and King, 1989; Lin and Spradling, 1995; McGrail and Hays, 1997; Máthé *et al*., 2003; review: **Chapter 1.2**). After the formation of the oocyte–nurse cell complex, the dividing mesodermal cells, associated with the *tunica propria* in region 2B, separate from this membrane and attach to the oocyte–nurse cell complex as follicle cells (also known as follicular epithelium cells; this term is used predominantly in this chapter). These eventually intercalate between the germ cells (Margolis and Spradling, 1995). The formation of the differentiated previtellogenic follicle (oocyte–nurse cell complex surrounded by the follicular epithelium cell monolayer) is completed within region 3, and the assembled follicle is expelled into the vitellarium. There it undergoes the transition from previtellogenesis to vitellogenesis and, subsequently, the transition to choriogenesis and ovulation.

During vitellogenesis, the oocyte receives material from the nurse and follicle cells and from the hemolymph. Some of these components represent differentially distributed determinants that establish pattern formation during early embryonic development, while others are nutrients necessary for the completion of embryogenesis. Upon completion of provisioning the oocyte and before the follicle is expelled into the oviduct, the follicle undergoes the transition into the choriogenic period. During this period, the oocyte is first sealed within a vitelline membrane before being covered by the chorion (see Section 1.3.3.8 below).

1.3.2.2. Lepidoptera

Many lepidopteran species have a relatively short, nonfeeding adult stage, which requires the adult female to emerge with most of her eggs ready to be fertilized and oviposited within hours. This life style constrains these insects to a program of ovarian organogenesis and follicle development that must occur at stages earlier than in *Drosophila*. Although the information base for ovarian and follicular cell development in the Lepidoptera is limited, this section will summarize the information that is available, and compare it with that obtained from *Drosophila*.

In the embryos of the domesticated silkworm, *Bombyx mori*, the primordial germ cells form a single cluster in the lateral and ventral region, which is associated with the localization of the *vasa* transcript (Nakao, 1999) rather than posteriorly, as in *Drosophila* (Hay *et al*., 1990; Lasko and Ashburner, 1990). The lateral location of the primordial germ cell cluster was suggested previously from electron microscopy observations (Miya, 1958, 1959) but, due to the absence of specific molecular markers, its position could not been confirmed. Nevertheless, the existing information suggests that the determinants required for germ cell formation are similar in moths and *Drosophila*, but there are spatial differences in their localization within the presumptive germ band.

The germ cells of *Manduca sexta* are first detected after germ band formation with a monoclonal antibody, 3B11, that specifically immunostains the large primordial germ cells (Nardi, 1993). These are observed as a loose aggregate in the midline of the embryo near the posterior pole, as is the case with *B. mori*, rather than directly posterior as in *Drosophila*. After segmentation of the embryo, the germ cells are distributed between three abdominal segments, and segregate laterally into two groups. Subsequently, the primordial germ cells form two tight clusters of 18–30 cells each that are localized within the fifth abdominal segment.

Ovary development in the Indian meal moth, *Plodia interpunctella*, was examined during the larval, pupal, and adult stages using an immunostain for the α-crystallin 25 kDa protein (αcp25), which is found primarily in the germ cells (Zimowska *et al*., 1991; Shirk and Zimowska, 1997; Shirk *et al*., 1998). In the newly hatched first instar *Plodia* larva, each gonad contains a single cluster of 28

germ cells (Beckemeyer and Shirk, in press). The gonads retain this morphology until the end of the first instar, when the germ cells of each ovary are segregated into four clusters of seven germ cells each (**Figure 3**, upper left; Beckemeyer and Shirk, in press). This segregation of the germ cells is completed in the early second instar, when the morphology of the four ovarioles within each gonad can be discerned (**Figure 3**, upper right; Beckemeyer and Shirk, in press). The organization of the ovarioles within the *Plodia* ovaries occurs much earlier than in *Drosophila*, where it begins at the end of the last larval instar. Whether the organization of the terminal filaments in *Plodia* is dictated by the localized expression of a *bab*-like gene within the undifferentiated somatic cells, as is the case with *Drosophila* (Godt and Laski, 1995), has yet to be determined.

No divisions of the primordial germ cells are observed until after the formation of the ovarioles is complete in the late second instar. Soon after the molt to the third instar, however, germ cell divisions begin, but there appears to be a separation between the seven founding primordial germ cells, which are maintained in the apical region of the ovarioles (**Figure 3**, lower left). The segregation of these seven cells may represent physically the niche of the germ stem cells in this moth. By the end of the penultimate instar, each ovariole contains about 56 germ cells.

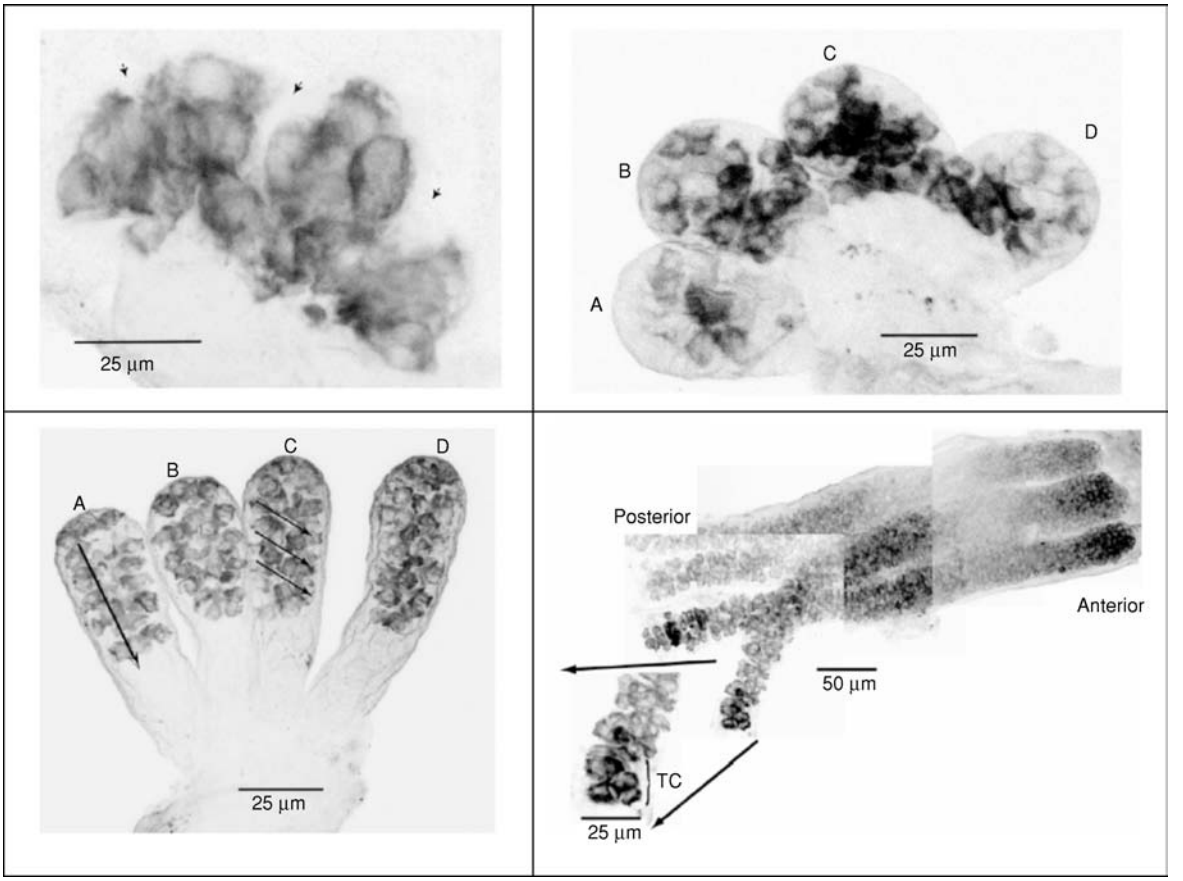


Figure 3 Upper left: a gonad from a late first instar *Plodia* larva immuno-fluorescently stained with α-crystallin (αcp25) antibodies. Somatic tissue segregation of the founding germ cells into separate ovarioles is apparent (marked by arrow heads). The confocal image is a compilation of optical sections through the specimen. Upper right: a gonad (ovary) from an early second instar *Plodia* larva stained with the same antibody. Segregation of the gonad into four ovarioles (A, B, C, and D) is complete and the number of cystoblasts has increased from the earlier stages. The confocal image is a compilation of optical sections through the specimen. Lower left: an ovary from a late third instar *Plodia* larva. The number of cystoblasts has increased and the cells show three levels of organization within the ovarioles. The confocal image is a compilation of half the optical sections through the specimen. The separation between the distal-most cluster of germ cells and the other germ cells within each ovariole is maintained. In ovariole A, a central space can be observed that does not contain immuno-fluorescently stained germ cells (paralleled by the half-open headed arrow). In ovariole C, a diagonal alignment of the germ cells can be seen (paralleled by the three arrows). Lower right: An ovary from a newly pupated *Plodia*. The image is a reassembly of three confocal scanning regions that cover the entirety of the ovary. Each region is the compilation of the optical sections through the region. The arrows show a projection of the most posterior or terminal clusters of cystocytes (TC).

The cluster of seven germ cells is maintained at the apical tip of each ovariole, while the majority of the germ cells are arranged in a spiral around the perimeter of the ovariole. No cystocyte clusters are observed at this stage, however, suggesting that in moths the divisions to form all of the primary cystoblasts occur before the onset of the cystocyte divisions. This is supported by the clear presence of eight-cell cystocyte clusters discernable in the posterior termini of the ovarioles at the end of the last instar (Zimowska *et al*., 1991; Beckemeyer and Shirk, in press).

The division of the cystoblasts and cystocytes in *Plodia* takes a path considerably different than that taken by *Drosophila*, where the primordial germ cells within the germarium give rise to the cystoblasts, which divide to form the 16-cell cystocyte cluster independent of the formation of other cystoblasts and throughout the adult stage (King *et al*., 1968). At the end of the third larval instar in *Plodia*, the divisions necessary to form all of the cystoblasts have taken place. This appears to happen before the three divisions of the cystoblasts to cystocytes begin. Thus, at the end of the third larval instar, the ovary contains all the cystoblasts necessary for the formation of the full complement of eggs that the female will lay during her adult life. Using a comparison to industry, *Plodia* mass-produces each stage of its germ cell/follicle production line before advancing to the next assembly stage, while *Drosophila* takes the on-time supply approach, by producing and assembling all components of a single follicle, given an adequate food supply and before initiating the production of a subsequent follicle.

The presence of the cluster of the seven primordial germ cells at the apical tip of each ovariole, even at the white pupa stage of *Plodia* (**Figure 3**, lower right) (Zimowska *et al*., 1991; Beckemeyer and Shirk, in press), suggests that there is a potential for producing more cystoblasts, even though all cystocyte clusters necessary for adult egg laying are already present. At this stage, however, the follicles are not complete, because no follicular epithelium cells are observed in association with the cystocyte clusters (Zimowska *et al*., 1991). Follicle cells begin to surround each oocyte–nurse cell complex during the ecdysteroid peak that occurs 28–36 h after pupation (Shaaya *et al*., 1993). By comparison, the organization of the ovarioles and germ cells in the early fifth (last) instar larva of *B. mori* (Miya *et al*., 1970) is similar to that observed in the white pupa of *P. interpunctella*, suggesting an even earlier completion of primordial germ cell development in *Bombyx*.

Following the peak of ecdysteroids that occurs during the early pupal stage (Shaaya *et al*., 1993), pharate adult development is initiated, and the terminal follicles become fully differentiated within the ovarioles of *Plodia* (Zimowska *et al*., 1991). The progress of the female toward the initiation of vitellogenesis is regulated by the declining levels of the ecdysteroids (Shirk *et al*., 1990). After final assembly by 36 h post-pupation, the follicles remain in a previtellogenic state until 92 h after pupation. After this time, the physiology of the female shifts to vitellogenesis, and each ovariole contains a linear array of sequentially developing follicles (**Figure 1**).

1.3.3. Vitellogenesis

During vitellogenesis, the growth of the oocyte in the ovarian follicle accelerates relative to that of the nurse cell complex (Büning, 1994). Growth of the oocyte is enhanced via endocytosis of yolk proteins, which may be derived from two sources, the fat body (vitellogenins) and the follicular epithelial cells that surround the oocyte–nurse cell complex (Raikhel and Dhadialla, 1992; Izumi *et al*., 1994; Melo *et al*., 2000). The oocyte surface is not directly exposed to the hemolymph but is separated from it by the follicular epithelium. Wide channels form between the epithelial cells, a condition termed patency (Telfer *et al*., 1982; Raikhel and Lea, 1991; Davey, 1993; Zimowska *et al*., 1994), allowing passage of hemolymph-derived proteins through the follicular epithelium toward the oocyte surface and into the oocyte.

1.3.3.1. The Transition from Previtellogenesis to Vitellogenesis

In this section, the hormonal control of the transition from previtellogenesis to vitellogenesis in the insects, mostly Diptera and Lepidoptera is discussed, that have been most intensely investigated, including the fruit fly *D. melanogaster*, the yellow fever mosquito, *Aedes aegypti*, and the silkmoth, *B. mori* is discussed. In these three insects, most of the data addressing the mechanism of action of hormones (predominantly ecdysone) in the ovarian tissue, has accumulated. The regulation of the transition in other insects will be only briefly discussed, using the models above as a basis for comparisons and, wherever possible, emphasis will be given to the molecular mechanisms controlling ovarian follicle development.

1.3.3.1.1. Dipteran insects

1.3.3.1.1.1. **Drosophila melanogaster** In *Drosophila*, previtellogenic growth of follicles, corresponding to stages 1–7 of egg chamber (follicle) development, is initiated at the end of adult development, while vitellogenesis (corresponding to

stages 8–10) starts after eclosion. The initiation of vitellogenesis constitutes a control point of oogenesis that integrates internal (hormonal) and external (environmental) signals, to allow efficient progression of follicle development (Spradling, 1993).

1.3.3.1.1.1.1. JH and ecdysone physiological data Hormonal control of vitellogenesis in *Drosophila* is complex and requires both JH (see **Chapter 3.7**) and ecdysteroids (Riddiford, 1993). JH stimulates yolk protein (YP) synthesis in the ovary, and is necessary for the continuation of development of stages 8 and 9 vitellogenic follicles (Jowett and Postlethwait, 1980; Wilson, 1982; Bownes *et al*., 1988a). In addition, JH stimulates the production of ovarian ecdysteroids (Schwartz *et al*., 1989).

Ecdysteroids play (see **Chapters 3.3**, **3.4**, and **3.7**) both positive and negative roles in the control of vitellogenesis. In diapausing female adults, oogenesis is arrested shortly before vitellogenesis but can be relieved by the application of 20-hydroxyecdysone (20E) (Richard *et al*., 1998). Based on this and other observations, it was proposed that in the early stages following adult eclosion, ecdysteroids derived from the ovary or from other sources (Bownes, 1989) stimulate YP synthesis in the fat body as well as YP uptake by the oocyte (Richard *et al*., 1998).

During the later stages of oogenesis, alternatively, 20E has a negative effect on follicle development. In virgin females, 20E induces apoptosis, causing the disappearance of nearly all vitellogenic follicles, and this effect can be counteracted by administration of JH or sex peptide (SP; Soller *et al*., 1999). The SP is a 36 amino acid long peptide synthesized by the accessory glands of *Drosophila* males, and transferred to the female fly during mating (Chen *et al*., 1988; Kubli, 1996) (see also **Chapters 1.5** and **3.9**). It acts on the corpora allata (CA) to stimulate the production of JH (Moshitzky *et al*., 1996), which, in turn, prevents the 20E-dependent induction of apoptosis in vitellogenic follicles. Thus, a correct balance between JH and ecdysteroids seems to be essential for the progression of vitellogenic follicle development in the *Drosophila* ovary (Soller *et al*., 1999; Gruntenko *et al*., 2003). The induction/prevention of apoptosis by 20E/JH shows strict developmental regulation and is restricted to follicles of stage 9, while follicles of earlier or later stages (stages 8 and 10, respectively) are not affected.

1.3.3.1.1.1.2. Genetic analysis role for ovarian ecdysone? Genes implicated in the ecdysone regulatory hierarchy show stage-specific expression patterns during *Drosophila* oogenesis. The levels of the mRNAs for the nuclear receptor E75 and the E twenty-six (ETS) domain-containing transcription factor E74 become upregulated at stage 8 (beginning of vitellogenesis) and peak in abundance at stage 10B (end of vitellogenesis), in both the nurse and the follicular epithelium cells (Buszczak *et al*., 1999). The E74 and E75 transcription factors function as primary response genes in the ecdysone regulatory cascade that is triggered at the beginning of metamorphosis (Thummel, 1996). Another primary ecdysone-responsive gene, which encodes the various Broad-Complex (BR-C) zinc finger protein-containing transcription factor isoforms, is also induced in the cells of the follicular epithelium during stages 5 and 6, and its expression continues throughout vitellogenesis (Deng and Bownes, 1997). Furthermore, both components of the ecdysone receptor heterodimer, EcR and USP, are expressed throughout oogenesis in both the germline (nurse cells and oocyte) and the soma (follicular epithelium cells) (Khoury-Christianson *et al*., 1992; Buszczak *et al*., 1999; Carney and Bender, 2000). Finally, the expression of *E75* and *BR-C* in the ovarian follicles seems to be sensitive to the levels of 20E: higher expression is observed when 20E is added to ovarian cultures, while lower expression occurs at the restrictive temperature in *ecdysoneless* (*ecd*) mutant flies (Buszczak *et al*., 1999).

Follicles that are germline clones of *E75* and *EcR* show developmental arrest and degeneration at stages 8–9 (Buszczak *et al*., 1999). A similar arrest in oogenesis was also observed for germline clones of the *dare* gene, which encodes the *Drosophila* homolog of adrenodoxin reductase, an enzyme implicated in steroid hormone biosynthesis (Buszczak *et al*., 1999; Freeman *et al*., 1999). Characterization of temperature-sensitive *ecdysoneless*^{1} (*ecd*^{1}) mutants, which have low ecdysteroid levels at the restrictive temperature, also indicate that 20E may be required for the progression of oogenesis beyond stage 8 (Audit-Lamour and Busson, 1981; Walker *et al*., 1987). Because *dare* is expressed in the nurse cells, it was proposed that *dare* is involved in ecdysone biosynthesis carried out by the nurse cell complex.

Based on the analysis of the available mutants and the expression pattern of genes implicated in ecdysone biosynthesis and the ecdysone regulatory hierarchy during oogenesis, a model was proposed in which ecdysteroids, produced by the germline oocyte–nurse cell complex at the beginning of vitellogenesis, act in an autocrine manner (i.e., within the developing follicle) to activate the ecdysone regulatory cascade. The activation of the ecdysone regulatory cascade is considered necessary for the subsequent progression of oogenesis (vitellogenesis)

beyond stages 8 and 9 (Buszczak *et al*., 1999; Freeman *et al*., 1999).

1.3.3.1.1.1.3. Integration of physiological and genetic data From the discussion above, it is clear that much work still needs to be done, in order to elucidate the precise role of ecdysone in the regulation of vitellogenesis in *Drosophila*. In particular, conflicting data exist between the results obtained from the physiological and genetic experiments that requires resolution. Indeed, the physiological experiments suggest that 20E stimulates previtellogenic growth, while it induces apoptosis of vitellogenic follicles (Richard *et al*., 1998; Soller *et al*., 1999; Gruntenko *et al*., 2003), and that a correct balance between ecdysteroids and JH is required for the progression through vitellogenesis (Soller *et al*., 1999). The genetic studies, alternatively, indicate a requirement for genes such as *EcR*, *E74*, *E75*, and *BR-C* in the transduction of the 20E response during metamorphosis (Buszczak *et al*., 1999; Carney and Bender, 2000). However, the fact that the *EcR*, *E74*, *E75*, and *BR-C* genes are involved in the control of oogenesis (vitellogenesis), does not necessitate that the regulatory pathway initiated by the hormone 20E is actually involved in this process. In fact, the data that support the model of autocrine control of follicle development progression, by the induction of the required regulatory cascade by 20E, may not be entirely conclusive.

Thus, the function of the *dare* gene may not be entirely limited to ecdysone biosynthesis, but could instead be involved in the catalysis of other enzymatic reactions. Of relevance to this is previous research in other insects, which has shown that the cells of the follicular epithelium, rather than the nurse cell complex, are involved in ecdysone biosynthesis (Lagueux *et al*., 1977). This has been confirmed through the expression pattern of the *disembodied* (*dis*) gene, which encodes a specific hydroxylase in the ecdysone biosynthetic pathway (Warren *et al*., 2002) and is expressed in the follicular epithelium (Chávez *et al*., 2000). The differential expression pattern suggests that the DARE and DIS enzymes are involved in distinct biosynthetic pathways. Therefore, it would be interesting to examine whether mutant clones for the *dis* gene (in the follicular epithelium) would affect the progression of vitellogenesis. Additonally, the fact that mutations of the *E75*, *E74*, *EcR*, and *BR-C* genes affect the progression of vitellogenesis, does not necessarily imply that the ecdysone regulatory pathway is involved. The expression of these genes could be regulated by other pathways. For example, during oogenesis, the expression of the *E75* and *BR-C* genes is controlled by the epidermal growth factor (EGF) pathway during oogenesis (Deng and Bownes, 1997; Buszczak *et al*., 1999). Furthermore, the requirements for *EcR* may reflect the function of this receptor as a repressor rather than as a ligand(20E)-dependent activator.

In view of these reflections, the view presented by the physiological experiments is favored, i.e., that 20E may be involved in the stimulation of previtellogenic growth but causes apoptosis in vitellogenic follicles. In addition, although genes implicated in the ecdysone regulatory pathway (*E75*, *E74*, *EcR*, and *BR-C*) are required for sustainance of vitellogenic growth, these genes may very well be induced and act in an ecdysone-independent manner.

1.3.3.1.1.1.4. Nutritional control through the insulin signaling pathway The quality of nutrition (absence/presence of protein diet) has profound effects on the rate of follicle development during oogenesis, which can vary by as much as 60-fold (Drummond-Barbosa and Spradling, 2001). Variations in the rates of follicle development are due to differences in rates of cell proliferation in both the germline and the follicle cell lineage, as well as to the different frequencies of cell death at two developmental stages, first, during egg chamber or follicle assembly, when the region of the germarium containing the germline cysts are engulfed by the precursors of the follicle cells, and, second, at the time of initiation of vitellogenesis (stage 8 of follicle maturation; Drummond-Barbosa and Spradling, 2001) (see also **Chapter 1.2**).

The insulin signaling pathway is conserved between *Drosophila* and vertebrates (Edgar, 1999), and *Drosophila* mutants for components of this pathway show a growth-deficiency phenotype (Garofalo, 2002). Mutants for the *Drosophila* insulin receptor, the insulin receptor substrate (IRS)-like gene *chico*, and the ribosomal protein S6 kinase homolog also show female sterility due to poor ovarian growth (Chen *et al*., 1996; Böhni *et al*., 1999; Montagne *et al*., 1999). These results suggest that the insulin signaling pathway is functional in the ovaries. Furthermore, the effects of nutrition on oogenesis are mediated through the insulin pathway. Thus, even in the presence of abundant nutrients, mutations in *chico* partially impair the increase in the proliferation rate of the follicular cells and almost completely prevent the progression of the follicles into vitellogenic stages (Drummond-Barbosa and Spradling, 2001).

Seven insulin-like peptide sequences have been identified in the *Drosophila* genome (DILPs), which show specific and unique patterns of expression in a variety of tissues, including the cells of the ovarian

follicular epithelium (Brogiolo *et al*., 2001; Ikeya *et al*., 2002). The expression levels of DILPs in the brain respond to variations in nutrition, and overexpression of DILP2 results in an increase in body weight and organismal size (Brogiolo *et al*., 2001). The picture that emerges is that signals generated by high nutrition levels result in the release of DILPs from neurosecretory cells at the corpora cardiaca (CC) of the ring gland (Leevers, 2001); DILPs secreted in the hemolymph subsequently stimulate cell proliferation and growth. At the level of the ovary, DILPs are proposed to increase the proliferation rate of the follicular cells and promote the transition from previtellogenesis to vitellogenesis. Furthermore, the expression of DILP5 in follicular cells of vitellogenic follicles (Ikeya *et al*., 2002) also suggests the existence of a parallel autocrine/paracrine pathway that regulates follicle growth and maturation.

With respect to the possible conservation of the signaling systems that regulate reproduction in insects and vertebrates, it is also worth mentioning that two orphan G protein-coupled receptors (GPCRs) have been identified in the *Drosophila* genome, which are homologs of the mammalian follicle stimulating hormone (FSH), luteinizing hormone (LH), and thyroid stimulating hormone (TSH) receptors (Hauser *et al*., 1997; Eriksen *et al*., 2000). However, no data exists for addressing the possible involvement of these receptors in the regulation of insect reproduction. In fact, flies mutant for one of these receptors die at the time of hatching, thus implicating this receptor in the control of embryonic development (Eriksen *et al*., 2000). Moreover, no FSH, LH, or TSH-like hormones could be identified in the *Drosophila* genome and, consequently, the two orphan *Drosophila* GPCRs may have other types of peptides as ligands (De Loof *et al*., 2001).

1.3.3.1.1.2. **Aedes aegypti** In contrast to the Lepidoptera (discussed in Section 1.3.3.1.2 below) and *Drosophila*, vitellogenesis and follicle maturation in female mosquitoes occur synchronously following a blood meal (Raikhel and Lea, 1990). At the time of adult eclosion, each ovary consists of the germarium and one follicle per ovariole. JH is required for the follicle to mature to a resting stage, at which time it becomes competent to respond to a blood meal and initiate vitellogenesis (Bownes, 1986). Vitellogenesis consists of two phases, first, of an initiation phase (immediately after the blood meal), during which the cells of the follicular epithelium resume mitosis and separate from the oocyte, while low levels of vitellogenins are produced by the ovary and, second, of a trophic phase (starting 5–6 h after the blood meal), during which the fat body produces large amounts of vitellogenins, and rapid growth of the follicle occurs in the ovary (Riehle and Brown, 2002).

During the initiation phase, neurosecretory hormones, termed egg development neurosecretory hormones (EDNHs) or ovary ecdysteroidogenic hormones (OEHs), are released from the brain and stimulate ecdysteroid production by the ovary (Brown *et al*., 1998; see also **Chapters 1.5** and **3.9**). The ecdysteroids released from the ovary subsequently initiate the trophic phase by acting on the fat body and stimulating the first vitellogenic cycle (Deitsch *et al*., 1995; Zhu *et al*., 2000). Ecdysteroids have also been proposed to be involved in a feedback mechanism that causes the pinching off of the second follicle from the germarium and the shutdown of the high synthetic activity in the fat body at the end of the cycle (Bownes, 1986).

As is the case with *Drosophila*, the insulin signaling pathway is involved in the regulation of ovarian growth (vitellogenesis). Mosquito ovaries contain high levels of the mosquito insulin receptor homolog (MIR; Graf *et al*., 1997). MIR is expressed in both the nurse and the follicular epithelium cells of the maturing follicles, and its expression levels in the cells of the follicular epithelium increase during the first phase of vitellogenesis (Helbling and Graf, 1998; Riehle and Brown, 2002).

Interestingly, the ovarian MIR can be stimulated by micromolar quantities of bovine insulin, a finding that allowed the elucidation of the signaling pathway in some detail. Bovine insulin was shown to be capable of stimulating tyrosine phosphorylation in the ovary (Riehle and Brown, 2002), and the downstream signaling was shown to involve the phosphatidyl-inositol 3-kinase (PI3K) and protein kinase B (PKB) pathways, but not that of the mitogen-activated protein kinase (MAPK) (Riehle and Brown, 1999, 2003). Furthermore, the action of bovine insulin results in the biosynthesis and release of ecdysteroids by the ovary (Riehle and Brown, 1999). Thus, insulin-like peptides have the same effect on ovarian tissue as the OEH hormones. To what extent the transduction pathways of these two types of hormones overlap or are integrated is unknown at present.

Although the ovary itself is not considered to be a direct target for 20E (Bownes, 1986), data exist with respect to the expression of ecdysone-responsive genes in the mosquito ovary. Of particular interest is the differential accumulation of various *usp* mRNA isoforms during previtellogenesis and vitellogenesis (Wang *et al*., 2000). During previtellogenesis, the mRNA for the A-isoform predominates, but this isoform becomes rapidly downregulated within 1 h after

a blood meal, and the decline persists throughout vitellogenesis. The mRNA for the USP-B isoform, alternatively, shows an opposite pattern of expression, becoming induced in the ovary after the blood meal. In addition, the expression of *EcR* mRNA increases at the initiation of vitellogenesis (Cho *et al*., 1995; Wang *et al*., 2000). Relevant to the differential regulation of target genes in follicular cells may be the observation that EcR/USP-B complexes bind more strongly than EcR/USP-A ones to target sites in the DNA, and are also stronger transcriptional activators in heterologous systems (Wang *et al*., 2000).

The expression patterns of mRNAs for early and early–late ecdysone response genes (*E75A*, *E75B*, *E75C*, *HR3*, and *E74B*) are nearly identical to that of the vitellogenin receptor (*VgR*) gene, suggesting a function for these genes in the regulation of vitellogenesis in the ovary (Pierceall *et al*., 1999; Kapitskaya *et al*., 2000; Sun *et al*., 2002). Although of unknown functional relevance, the B isoform of the mosquito HNF-4 nuclear receptor (see also discussion on lepidopteran HNF-4 expression below) is also present in ovarian tissue, and its expression is induced following a blood meal (Kapitskaya *et al*., 1998).

Taken together, these results suggest that genes historically implicated in the ecdysone response have an expression pattern consistent with their having roles as regulators of vitellogenesis in the ovary. Whether the expression of these genes in the ovary is regulated by ecdysteroids, however, remains to be investigated. As was discussed for the case of *Drosophila* (Section 1.3.3.1.1.1.3), the expression of the "ecdysone response" genes may be under the control of other signal transduction pathways.

While uncertainty exists in the case of ovarian tissue, the induction of early and early–late response genes by 20E in the fat body has been demonstrated beyond doubt, and additional data suggest that the regulatory cascade induced by 20E in the fat body of the adult female shows great resemblance to the cascade induced by 20E in other tissues during metamorphosis (Raikhel *et al*., 1999; Li *et al*., 2000; Zhu *et al*., 2000).

1.3.3.1.2. Lepidoptera Lepidopterans can be grouped in different categories based on their dependence for ovarian follicle maturation on metamorphic events (Ramaswamy *et al*., 1997). The silkmoth *B. mori* exemplifies the first category, in which follicle development is dependent on 20E, occurs during pupal and adult development, and is completed before the emergence of the adult. The second category, is typified by the Indian meal moth, *P. interpunctella*, in which vitellogenesis is triggered by the declining titers of ecdysteroids during adult development (Shirk *et al*., 1992). Follicle development in this category is also completed before adult eclosion. In a third category, exemplified by the tobacco hornworm, *M. sexta*, initiation of vitellogenesis during metamorphosis is independent of ecdysteroids (Satyanaryana *et al*., 1994), while choriogenesis is controlled by JH and completed in the adult moth (Nijhout and Riddiford, 1974).

In other lepidopterans such as *Heliothis virescens* (fourth category), the initiation and completion of vitellogenesis and choriogenesis are independent of metamorphic events, with follicle development being initiated in adults and completely dependent on JH (Zeng *et al*., 1997). Finally, an additional version of hormonal control has been described recently in the fall armyworm, *Spodoptera frugiperda* (Sorge *et al*., 2000). In the adults of this species, JH is crucial for the regulation of the incorporation of yolk proteins in vitellogenic follicles, and for the induction of ecdysteroid production by the ovary, in a manner reminiscent of the one occurring in *Drosophila*. Ecdysteroids produced by the ovary in turn regulate the production of vitellogenins by the fat body (Sorge *et al*., 2000).

***1.3.3.1.2.1.* Bombyx mori** In *Bombyx*, oogenesis occurs almost exclusively during pupal and pharate adult development and is completed before adult eclosion (Tsuchida *et al*., 1987). The moulting hormone 20E was shown to be essential and sufficient for initiating previtellogenic and vitellogenic development in ovarian follicles. Thus, when late spinning larvae are ligated between the thorax and the abdomen, 20E that is produced by and secreted from the prothoracic glands is prevented from entering the abdominal cavity, and developmental arrest ensues. Adult development, including ovarian development, can be induced in the arrested abdomens by injections of microgram quantities of 20E (**Figure 4**) (Tsuchida *et al*., 1987; Swevers and Iatrou, 1999).

The other major hormone that regulates development in insects, JH, has not been found to be involved in the stimulation of ovarian development in *Bombyx*. Thus, allatectomy of the 4th instar larvae does not prevent ovarian development and egg maturation (vitellogenesis and choriogenesis) in the resulting precocious pupae, suggesting that JH does not play a role during oogenesis (Izumi *et al*., 1984).

The rise in ecdysteroid titer, during pupal and pharate adult development, initially results in important morphological changes in the ovarian structure. The ovarian capsule, in which the ovarioles are assembled and enclosed during larval and pupal development, ruptures and the developing ovarioles emerge gradually in the abdominal cavity

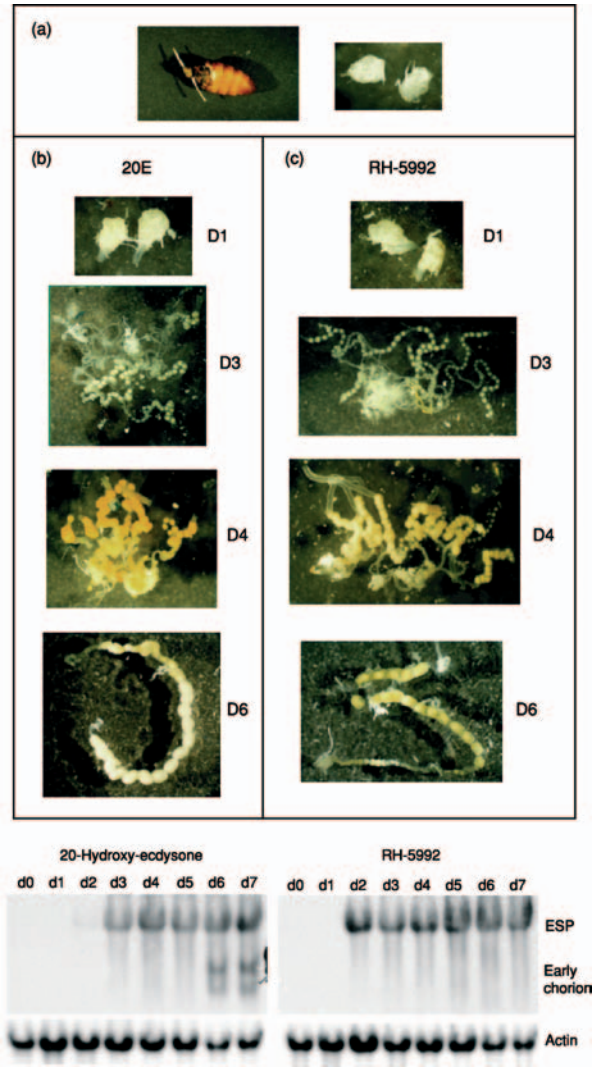


Figure 4 The nonsteroidal ecdysone agonist RH-5992 (tebufenozide) induces ovarian development (previtellogenic growth and vitellogenesis) in the silkmoth, *Bombyx mori*, that is followed by developmental arrest prior to choriogenesis. Upper panel: photographs of a developmentally arrested abdomen (panel (a), left) and of ovaries and ovarioles from abdomens that were either uninjected (panel (a), right), injected with 20E (panel (b)), or injected with RH-5992 (panel (c)). Ovaries and ovarioles are either in dissection medium (panel (a), right, and series D1, D3, and D4 from panels (b) and (c) or mounted in glycerol (D6 in panels (b) and (c). Indicated are the time intervals (in days, D) between injection and dissection. Note that for ovarioles mounted in glycerol, oocytes in choriogenic follicles are prevented from collapsing by the protective eggshell. Color also distinguishes vitellogenic (yellow) from choriogenic (white) follicles. Choriogenic follicles are only apparent in D6 ovarioles from 20E-injected abdomens. Lower panel: expression of egg-specific protein (ESP) and early chorion mRNAs in ovaries at different time intervals (in days, d) following injection of 20E or RH-5992 (tebufenozide) in pupal developmentally-arrested abdomens of *B. mori*. Hybridizing mRNA species in Northern blot analysis are 2 kb (ESP), 0.8 and 0.6 kb (early chorion), and 1.3 kb (actin). Note that ESP mRNA is induced in both 20E- and tebufenozide-injected abdomens, while early chorion mRNA only appears in the former. (Reprinted with permission from Swevers, L., Iatrou, K., **1999**. The ecdysone agonist tebufenozide (RH-5992) blocks progression of the ecdysteroid-regulated gene expression cascade during silkmoth oogenesis and causes developmental arrest at mid-vitellogenesis. *Insect Biochem. Mol. Biol.* 29, 955–963; © Elsevier.)

(see **Figure 12** below) (Yamauchi and Yoshitake, 1984). The removal of the ovarian capsule allows the follicles in the ovarioles to take up yolk proteins from the hemolymph and facilitates their direct exposure to growth factors that are found in it.

At the molecular level, the silkmoth ovary responds to the circulating 20E through the induction of a gene expression cascade that is practically identical to the one observed in other insect tissues (the ecdysone regulatory cascade; Henrich and Brown, 1995; Thummel, 1996). The ovarian tissue expresses the nuclear receptors that constitute the functional ecdysone receptor, which is composed of the heterodimer complex between EcR and USP (Tzertzinis *et al.*, 1994; Swevers *et al.*, 1995), suggesting that the ovary is a direct target of 20E. Similar to other

insect tissues, 20E induces, within a few hours, the expression of the primary response genes, which encode the nuclear receptors BmHR3 (B and C isoforms) and BmE75 (A and C isoforms) (Eystathioy *et al*., 2001; Swevers *et al*., 2002a). During the induction of expression of the mRNAs for these receptors, the mRNA for another nuclear receptor, BmFTZ-F1 (Sun *et al*., 1994), becomes down-regulated (Swevers and Iatrou, 2003).

The expression of a sixth nuclear receptor, BmHNF-4, is also induced in ovarian tissue (follicular epithelium cells) upon exposure to 20E (Swevers and Iatrou, 1998). In contrast to the BmE75 and BmHR3 receptors, however, the accumulation of BmHNF-4 occurs more slowly and appears to represent a delayed response to the hormone. To date, BmHNF-4 has not been associated with the induction of the ecdysone response in other tissues (Thummel, 1995).

The expression of the yolk protein, egg-specific protein (ESP; Sato and Yamashita, 1991), which is produced by the cells of the follicular epithelium, is initiated approximately two days after larval–pupal ecdysis, when ecdysteroid titers reach peak levels of accumulation in the hemolymph (Tsuchida *et al*., 1987), and the time of first expression of ESP can be viewed as a timing marker for the initiation of vitellogenesis (Sato and Yamashita, 1991). Moreover, when 20E is injected in developmentally arrested abdomens, ESP expression occurs with similar kinetics as during normal development (2–3 days after injection; **Figure 4**; Swevers and Iatrou, 1999), indicating that ESP expression represents a late response in the ecdysone regulatory cascade. The expression of the *esp* gene does not seem to be directly regulated by 20E, however, because it can occur both during the period of ecdysteroid accumulation to high titers (normal development) and in the *quasi* absence of 20E (developmentally arrested abdomens injected with 20E, in which the hormone is neutralized within 24 h from the injection; Ohnishi and Chatani, 1977).

The expression of two types of transcription factors, BmHR3 and BmGATAβ, shows remarkable changes in abundance in ovarian follicles at the transition from previtellogenesis to vitellogenesis. The A isoform of the nuclear receptor BmHR3 becomes expressed in the cells of the follicular epithelium of vitellogenic follicles (Eystathioy *et al*., 2001), while there is also a remarkable increase in the accumulation of the mRNAs for the other two isoforms of BmHR3, B and C. The expression of BmHR3 and ESP during vitellogenesis coincides remarkably, and retinoic acid receptor-related orphan receptor response elements (ROREs) capable of binding BmHR3A have been identified in the *esp* gene promoter (Eystathioy *et al*., 2001), implicating BmHR3A as a potential regulator of *esp* expression (Swevers and Iatrou, 2003). In addition, the expression of the zinc finger-containing transcription factor BmGATAβ (Drevet *et al*., 1994) is down-regulated during the transition from previtellogenesis to vitellogenesis. In this case, however, immunocytochemistry experiments have shown that expression of BmGATAβ is restricted to the cells of the ovariole sheath rather than the follicular epithelium (Lunke, 2000). Thus, while the expression of BmGATAβ has been correlated with the transcriptional competence of chorion genes (see Sections 1.3.3.3.2 and 1.3.4.1.3), it does not seem to play a role in the differentiation of the follicular epithelium during the transition from previtellogenesis to vitellogenesis. Instead, it may have a specialized (but as yet undefined) role in the ovariole sheath cells that overlay the previtellogenic follicles (Swevers and Iatrou, 2003).

1.3.3.1.2.2. **Plodia interpunctella** Vitellogenesis in *Plodia interpunctella* begins during the pupal stage, which lasts 136 h, and eggs are completely chorionated and ready for fertilization by 6 h after adult eclosion (Zimowska *et al*., 1991). As is typical of most Lepidoptera, each of the four ovarioles within the ovary contains a linear array of progressively developing follicles, with the most posterior or terminal follicle being the most advanced in development.

During vitellogenesis, oocytes accumulate two major yolk proteins: vitellin (Vn), which consists of two yolk protein (YP) subunits, YP1 ($M_r = 153\,000$) and YP3 ($M_r = 43\,000$), produced and secreted as vitellogenin (Vg) by the fat body cells (see also Section 1.3.3.2 below); and follicular epithelium yolk protein (FEYP), which consists of two subunits, YP2 ($M_r = 69\,000$) and YP4 ($M_r = 33\,000$), produced and secreted by the follicular epithelium cells (Shirk *et al*., 1984; Bean *et al*., 1988). The presence of vitellogenin in the hemolymph can be detected by immuno-staining of Western blots with antisera for YP1 or YP3 at 80–83 h after pupation (Shirk *et al*., 1992). However, Vn cannot be detected by immuno-gold staining with anti-YP1 or anti-YP3 antisera in the terminal follicles until after 112 h after pupation (Zimowska *et al*., 1994, 1995). Repeated treatments of pharate adult female pupae with 20E leads to the suppression of the oocyte growth and yolk protein accumulation (Shirk *et al*., 1990), suggesting that the developmental progress of female *P. interpunctella* toward vitellogenesis is regulated by the declining titers of ecdysteroids.

Between the previtellogenic and vitellogenic stages, the terminal follicles enter the provitellogenic stage, during which the follicles become competent to produce FEYP within the follicular epithelium cells, initiate the formation of yolk spheres, and develop patency, the opening of spaces between the follicular epithelium cells that allows penetration of the hemolymph proteins into the oocyte (Zimowska *et al*., 1994). Using immuno-gold staining with anti-YP2 serum, it was established that the follicular epithelium cells begin production of YP2 at 92 h after pupation and, at the same time, the oocytes begin formation of YP2-containing yolk spheres (Zimowska *et al*., 1994). However, none of the other yolk protein subunits is detectable at this time. By culturing early vitellogenic ovaries in varying concentrations of 20E, it was shown that the continuation of YP2 synthesis requires low concentrations of 20E but is inhibited by high levels of the hormone (Shirk *et al*., 1990). These findings suggest that the action of the ecdysteroids is to regulate the progression of vitellogenesis by acting directly on the synthetic activity of the ovarian tissue.

The formation and accumulation of early yolk spheres containing only YP2 and the increased thickness of the brush borders of the follicular cells are both indicators of the progression towards vitellogenesis (Zimowska *et al*., 1994). By 105 h after pupation, the follicular epithelium of the terminal follicles begins to show signs of patency. By 120 h after pupation, full patency is achieved, and both yolk proteins, Vn and FEYP, are accumulated in the yolk spheres (Zimowska *et al*., 1994, 1995; see also Section 1.3.3.2).

1.3.3.1.2.3. Other Lepidoptera While progress has been made regarding the effects of 20E on the silkmoth ovary at the molecular level, no such data exist regarding the hormonal control of vitellogenesis by JH or 20E in the other categories of lepidopterans mentioned earlier. It can be anticipated, however, that regulatory cascades other than the ecdysone cascade are involved. For instance, in the case in which vitellogenesis is triggered by a decline in ecdysteroid titer (*P. interpunctella*; Shirk *et al*., 1992), the involvement of the nuclear receptor FTZ-F1 is expected, since this receptor functions as a competence factor during developmental periods characterized by low ecdysteroid titers (Broadus *et al*., 1999). However, regarding the response to JH, little is known, and a "JH-induced regulatory cascade" has yet to be characterized (Gilbert *et al*., 2000). It remains to be seen to what extent the regulation of vitellogenesis by 20E or JH during follicle development, in lepidopterans belonging to the different categories mentioned above, is carried out by common regulatory factors. It is obvious, however, that no unifying model for the control of the transition from previtellogenesis to vitellogenesis can be proposed at the moment.

1.3.3.1.3. Other insects In other insects where oogenesis has been studied extensively, such as the African migratory locust, *Locusta migratoria*, the cockroaches *Blattella germanica* and *Leucophaea maderae*, and the hemipteran, *Rhodnius prolixus*, JH has been implicated as the major hormone for the control of vitellogenesis (Bownes, 1986; Wyatt, 1991; Davey, 1993; Cruz *et al*., 2003). However, very little information exists with respect to the action of JH in developing follicles at the molecular level. In addition to the action of JH, a few peptide hormones have been also isolated that stimulate or inhibit vitellogenesis (De Loof *et al*., 2001).

The action of JH on ovarian follicle cells has been elucidated to the greatest extent in *R. prolixus* (Davey, 2000). In *Rhodnius*, as in many other insects, JH is essential for vitellogenin uptake by the oocyte. The target cells in the ovary are the cells of the follicular epithelium: the action of JH causes a great reduction in their cellular volumes, and this results in the creation of intercellular channels between the follicular cells (patency), which allows passage of vitellogenin from the hemolymph toward the oocyte surface (Telfer and Woodruff, 2002). At the molecular level, JH acts by binding to an as yet undefined membrane receptor, the subsequent activation of calcium-dependent protein kinase C, and phosphorylation of a polypeptide resembling in size the 100 kDa subunit of Na^+/K^+-ATPase (Sevala and Davey, 1993; Sevala *et al*., 1995). Presumably this leads to a change in the osmotic pressure of the follicular epithelium cells, concomitant shrinkage of their volume, and establishment of patency.

In addition to its action at the membrane level, JH has effects also at the transcription level in the cells of the follicular epithelium. In *B. germanica*, JH stimulates the accumulation of transcripts for the Ca^{2+}-dependent regulatory protein Calmodulin (CAM) in the cells of the follicular epithelium (Iyengar and Kunkel, 1995). The mechanism of action of JH at the level of regulation of gene transcription in the nucleus is unknown at present (Gilbert *et al*., 2000).

In *L. migratoria*, a peptide gonadotropic hormone, called ovary maturating parsin (OMP), has been identified, which is active during the same period of vitellogenesis as JH (Girardie *et al*., 1991; see also **Chapters 1.5** and **3.9**). OMP, however, acts independently of JH, since it is capable of inducing

vitellogenesis in allatectomized animals (Girardie *et al*., 1996). OMP stimulates vitellogenesis via two distinct hydrophilic domains (Girardie *et al*., 1998a). On the one hand the C-terminal part presumably acts on ovarian tissue to stimulate ovarian ecdysteroidogenesis, with the produced ecdysteroids subsequently stimulating vitellogenin production by the fat body and concomitant ovarian growth (Girardie and Girardie, 1996; Girardie *et al*., 1998a). The N-terminal part of OMP, on the other hand, acts on the fat body and stimulates vitellogenin production by increasing its mRNA stability and translatability (Girardie *et al*., 1998a). The mode of action of either domain of OMP at the molecular level is unknown.

Other peptides derived from neural tissue that may play a role in the regulation of ovarian growth include neuropeptide F-like peptides in locusts and a beetle (Cerstiaens *et al*., 1999), neuroparsins in locusts (Girardie *et al*., 1998b; Janssen *et al*., 2001), and a cAMP-generating peptide in fleshflies (Schoofs *et al*., 1994). Neuropeptide F-like peptides stimulate ovarian growth and act through GPCRs (Mertens *et al*., 2002). Neuroparsins, however, have anti-JH effects and are inhibitory to ovarian growth (Girardie *et al*., 1998b). For both the neuropeptide F-like peptides and the neuroparsins, it is not known whether the ovary is the primary target organ. The cAMP-generating peptide alternatively, has been isolated by its capacity to enhance cAMP production in the ovaries of the flesh fly, *Neobellieria bullata* (Schoofs *et al*., 1994), but no data exists regarding a correlation between cAMP levels and ovarian differentiation in this species.

Recently, a second type of neuroparsin has been identified in locusts, whose expression in the brain is upregulated during the gonadotropic cycle (Janssen *et al*., 2001). Because of its expression pattern, this second type of neuroparsin may act as a stimulator, rather than an inhibitor, of ovarian development. It should also be noted that locust neuroparsins show homology to the ovary ecdysteroidogenic hormone (OEH) of the mosquito, which has gonadotropic and ecdysteroidogenic activity on ovarian tissue (Brown *et al*., 1998; Janssen *et al*., 2001). As can be hypothesized for mosquito OEH, neuroparsins may function as modulators (negative or positive, dependent on type) of the putative insulin response in locusts.

From the discussion above, it is evident that oogenesis in insects is not regulated by a single, general hormonal mechanism. For all insects, an involvement of at least one of the two major insect morphogenetic hormones, 20E and JH, is apparent. However, the relative contribution of these hormones to the control of oogenesis differs from species to species. Furthermore, the action of 20E or JH in different insect species is exerted on different control points of oogenesis.

More recently, the involvement of neuropeptides in the control of oogenesis could be clearly demonstrated, but again no common "master neuroregulatory circuit" could be identified that is conserved in all insects. This contrasts the situation in vertebrates, where the control of ovarian development is carried out by a conserved set of hormones such as gonadotropins and sex steroids.

1.3.3.2. Ovarian Yolk Proteins

The fat body is the principal site of production of the major yolk protein precursors (YPPs), which are vitellogenin (Vg) in most insects, and yolk peptides (YPs) in cyclorrhaphan (higher) Diptera. It is also the production site of additional YPPs, which are synthesized in a unique fashion in different insect species depending on their life histories and developmental requirements. Remarkably, considering the extreme specialization of this tissue, the fat body of several insects also produces pro-proteases, which are deposited in the developing oocytes. These proteolytic enzymes are involved in the degradation of yolk proteins during embryogenesis.

In most insects, the major protein component of the yolk is vitellin (Vn), the crystallized storage form of Vg. Insect Vgs are large, conjugated proteins. They are translated as primary products with masses of ~200 kDa, which are usually cleaved into subunits (apoproteins) ranging in size from 50 to 180 kDa, although cleavage is not universal (Sappington and Raikhel, 1998b; Maruta *et al*., 2002; Sappington *et al*., 2002). Following extensive co- and posttranslational modification, the Vg subunits form high molecular weight oligomeric phospholipoglycoproteins (400–700 kDa), which are secreted into the hemolymph of vitellogenic females (Sappington and Raikhel, 1998b; Tufail *et al*., 2004). For example, in *P. interpunctella*, the major yolk protein component, Vn, is comprised of two subunits, YP1 ($M_r = 153$ kDa) and YP3 ($M_r = 43$ kDa). It is produced and secreted as Vg by the fat body cells and accumulates in the oocyte during vitellogenesis (Shirk *et al*., 1984; Bean *et al*., 1988).

In cyclorrhaphan Diptera such as *Drosophila*, the major YPPs belong to a class of proteins evolutionarily distinct from Vg (Sappington and Raikhel, 1998b; Sappington, 2002), which is not produced at all. Designated "yolk polypeptides" or YPs, they are relatively small proteins (~45 kDa) that are glycosylated, phosphorylated, and sulfated (Bownes and Pathirana, 2002). Interestingly, the YPs of

cyclorrhaphan Diptera are evolutionarily related to *B. mori* ESP and the YP2 subunit of the follicular epithelium yolk protein (FEYP) of pyralid moths, both YPPs that are produced as supplements to Vg (Inagaki and Yamashita 1989; Shirk and Perera 1998; Sappington 2002). They are also homologous to the mammalian triacylglycerol lipase, but lack the functional enzymatic center (Baker and Wolfner, 1988; Bownes *et al*., 1988b; Terpstra and Ab, 1988; Bownes, 1992).

A number of supplemental proteins have been described that accumulate selectively in developing oocytes. Some of them originate from the fat body and some from the cells of the follicular epithelium. These proteins include both female-specific and bisexually distributed members and, normally, they represent minor components of the yolk. For example, eggs of the stick insect, *Carausius morosus*, contain a minor YP that is secreted by the fat body and sulfated by the follicle cells (Giorgi *et al*., 1995). However, in a few cases they are almost as abundant as vitellogenin. Thus, in *P. interpunctella*, FEYP, which is comprised of two subunits, YP2 ($M_r = 69$ kDa) and YP4 ($M_r = 33$ kDa), is produced and secreted by the follicular epithelium cells and taken up by the oocyte (Shirk *et al*., 1984; Bean *et al*., 1988; see also Section 1.3.3.1.1.2 above). Though not as abundant as Vg, it comprises 25–40% of the yolk protein (Shirk and Perera, 1998). A 30 kDa YPP, called microvitellogenin, is produced by the fat body in *Hyalophora cecropia* and *M. sexta* (Kawooya *et al*., 1986; Telfer and Pan, 1988; Pan *et al*., 1994). The fat body of the domesticated silkworm *B. mori* also produces multiple 30 kDa proteins homologous to microvitellogenin (Wang *et al*., 1989). These proteins are principal components of the hemolymph of both males and females during the late larval and pupal stages of this insect (Izumi *et al*., 1981). The 30 kDa proteins of *Bombyx* constitute 35% of the egg's total soluble protein, while Vn comprises 40% (Zhu *et al*., 1986). In *L. migratoria* females, a 21 kDa and a 25 kDa protein are produced by the fat body in synchrony with the synthesis and secretion of Vg (Zhang *et al*., 1993; Zhang and Wyatt, 1996). These additional YPPs are significant constituents of the yolk in *Locusta* eggs, with the molar concentration of the 21 kDa protein in eggs being close to that of Vn.

Lipids are a critical source of energy during insect embryogenesis (Beenakkers *et al*., 1985; Van Antwerpen *et al*., 2004). They represent 30–40% of the insect egg's dry weight (Troy *et al*., 1975; Kawooya and Law, 1988; Briegel, 1990). Although Vg has a lipid content of 8–15%, its contribution to the total lipid content of the egg apparently is minor (Kawooya *et al*., 1988; Maruta *et al*., 2002; Van Antwerpen *et al*., 2004). The role of lipophorin (Lp), the insect lipid transport protein, in lipid accumulation in insect oocytes is much greater, and was recently reviewed by Van Antwerpen *et al*. (2004). In addition to its role as a lipid carrier, Lp plays a role as a YPP. In saturniid and sphingid moths, Lp is the second most abundant YP behind Vg (Telfer and Pan, 1988). Lipophorin is also present in the yolk of the fall webworm, *Hyphantria cunea* (Yun *et al*., 1994), and the mosquito *A. aegypti* (Sun *et al*., 2000).

Biochemical and molecular characterization of two female-specific, fat body-secreted proteins that are deposited in the yolk of *A. aegypti* mosquitoes has revealed that they are proenzymes. The first is a glycosylated 53 kDa protein secreted by the female fat body and accumulated in the developing oocyte in synchrony with Vg (Hays and Raikhel, 1990; Snigirevskaya *et al*., 1997a). Analysis of the deduced amino acid sequence has shown that it is the proenzyme for a serine carboxypeptidase. This stored proenzyme, vitellogenic carboxypeptidase (VCP), is activated during embryogenesis by cleavage to a 48 kDa polypeptide (Cho *et al*., 1991). The second protein, a 44 kDa fat body product with sequence similarities to vertebrate cathepsin B, is also incorporated into the yolk of *A. aegypti* (Cho *et al*., 1999). It is converted to a 42 kDa protein after internalization in the developing oocytes, and further reduced to 33 kDa in developing embryos, coincident with the onset of Vn degradation. The 33 kDa peptide exhibits enzymatic properties of a thiol cathepsin B-like protease, and has been shown to degrade purified mosquito Vn; hence it was named vitellogenic cathepsin B (VCB) (Cho *et al*., 1999).

An acid cysteine proteinase purified from *B. mori* eggs was subsequently named *Bombyx* cysteine proteinase (BCP). It has a broad substrate specificity and hydrolyzes yolk proteins. The proform of BCP accumulates in the hemolymph, suggesting a fat body origin. Indeed, Northern blot analysis clearly showed that *bcp* mRNA is expressed in the fat body (Kageyama and Takahashi, 1990; Yamahama *et al*., 2004).

In several studied insects, many other proteins are incorporated into the yolk (Telfer, 2002; Masuda *et al*., 2004). For example, the iron transport protein transferrin (~65 kDa) is selectively deposited in the yolk of the flesh fly, *Sarcophaga peregrina* (Kurama *et al*., 1995), and a similar protein is deposited in the bean bug *Riptortus clavatus* (Hirai *et al*., 2000). The hemolymph and oocytes of *R. prolixus* also contain a 15 kDa heme-binding protein (Oliveira *et al*., 1995). In Lepidoptera, a blue biliprotein

called insecticyanin, a component of larval hemolymph, is deposited in developing oocytes and is present in mature eggs (Chino *et al*., 1969; Kang *et al*., 1995). Biliverdin is a chromophore covalently bound to Vg in *Spodoptera litura*, coloring the eggs green or blue, presumably providing camouflage against green vegetation (Maruta *et al*., 2002). Finally, an arylphorin-like cyanoprotein, which is a hemolymph storage hexamer, also is deposited in eggs of the bean bug, *R. clavatus*, as a YPP (Chinzei *et al*., 1990; Miura *et al*., 1994). For more detailed information on insect yolk proteins and their precursors, see Bownes and Pathirana (2002), Sappington *et al*. (2002), Telfer (2002), Masuda *et al*. (2004), Tufail *et al*. (2004), and Yamahama *et al*. (2004).

1.3.3.2.1. Yolk protein gene transcription factors
In *Drosophila*, the gene structures of the three yolk protein genes, *yp1–3*, have been elucidated. *Cis*-regulatory elements were determined in the promoter regions, which are necessary for expression in ovarian follicular cells and in the fat body (Logan and Wensink, 1990; An and Wensink, 1995). Almost all studies have focused on the *yp1/yp2* gene pair whose members are divergently transcribed from a common promoter region. Only very recently has the analysis been extended to the unlinked *yp3* gene (Hutson and Bownes, 2003). To date, most studies have focused on the regulation of these genes in the fat body, with only a limited number of studies addressing their regulation in the ovary.

With regard to expression in the ovarian follicular epithelium cells, genetic analysis in transgenic flies identified two enhancer regions, ovarian enhancer 1 (OE1) and OE2, that are located in the intergenic region and the first exon of *yp2*, respectively (Logan *et al*., 1989; Logan and Wensink, 1990). The OE regions are distinct from the fat body enhancer (FBE) region, which mediates expression of the yolk protein genes in this tissue (An and Wensink, 1995). The *trans*-acting factors that regulate transcription through OE1 or OE2 have yet to be identified.

Biochemical methods, such as gel retardation assays and *in vitro* transcription assays, have revealed two *trans*-acting factors implicated in the regulation of yolk protein expression in follicular cells. The first factor, yolk protein factor 1 (YPF1), is a homolog of the Ku autoantigen that corresponds to the DNA-binding subunit of DNA-dependent protein kinase (Mitsis and Wensink, 1989; Jacoby and Wensink, 1994). YPF1 binds to a 31 bp sequence located immediately downstream of the *yp1* transcription start site; deletion of this site results in a marked decrease in expression levels of *yp1* mRNA, suggesting that YPF1 is a regulator of *yp1* gene expression (Jacoby and Wensink, 1994). The expression of *ypf1* mRNA is upregulated in egg chambers during vitellogenesis and choriogenesis (Jacoby and Wensink, 1994).

The second factor, dGATAb, belongs to the GATA family of transcriptional activators. An ovary-specific isoform of dGATAb is specifically expressed in the follicular epithelium cells and functions as stimulator of yolk protein gene expression through binding to a palindromic GATA element in the intergenic region of *yp1* and *yp2* (Lossky and Wensink, 1995). It has been proposed that dGATAb is not a stimulatory transcription factor by itself, but rather acts through the formation of partnerships with other proteins, presumably the ones that bind the OE1 and OE2 enhancers (Lossky and Wensink, 1995).

In contrast to the fat body, expression of yolk protein genes in the follicular epithelium cells is not dependent on the sex determination hierarchy (Wolfner, 1988). Experiments using a temperature-sensitive mutation of *transformer-2* (*tra-2*), a component in the sex determination hierarchy, have shown that this hierarchy (see **Chapter 1.1**) functions only during the formation of the ovary. Once the ovarian follicle cells have formed, however, expression of female-specific genes (such as the *yp* genes) in the ovary is regulated by tissue (ovary)-specific factors and is independent of the sex determination hierarchy (Bownes *et al*., 1990).

In *B. mori*, the orphan nuclear receptor BmHR3A is expressed in the cells of the follicular epithelium of vitellogenic follicles concomitant with the expression of the follicle cell-specific yolk protein ESP (see Section 1.3.3.1.2.1), an analog of *Drosophila* YPs (Sappington, 2002). BmHR3A functions as a transcriptional activator in tissue culture cells and a HR3-binding element has been identified within the *esp* promoter (Eystathioy *et al*., 2001). It should also be noted that the mosquito AaHR3 is expressed in the ovaries during vitellogenesis together with other transcriptional regulators implicated in the ecdysone regulatory hierarchy. Thus, although functional studies are lacking, the data suggest that HR3 may be a regulator of follicle cell yolk protein gene expression in insects.

1.3.3.2.2. Receptor-mediated endocytosis of yolk proteins in insect oocytes
1.3.3.2.2.1. Early studies of yolk protein uptake in insect oocytes Insect oocytes accumulate enormous amounts of yolk protein precursors that are mainly produced by the fat body. In turn, oocytes are extraordinarily specialized for the specific uptake of these proteins. The first observations related

to the specific accumulation of YPPs were made by Telfer (1960) in the developing oocytes of the silkmoth, *Hyalophora cecropia*, while the first interpretation of endocytosis came from electron microscopic studies of the developing oocytes of the mosquito *A. aegypti* (Roth and Porter, 1964). This latter study revealed the presence of a large number of membrane pits and vesicles in the cortical layer of the oocyte cytoplasm of female mosquitoes after a blood meal. Roth and Porter (1964) described a "bristle layer" on the cytoplasmic side of these vesicles, referred to these vesicles as "coated," and attributed their presence to YPP uptake. They also suggested that coated vesicles carrying YPPs lose their bristle coat, form larger vacuoles, and then grow into "protein droplets," the yolk bodies (**Figure 5**).

Numerous electron microscopic studies of developing oocytes in various insect orders have subsequently shown the presence of these vesicular compartments – coated pits, coated vesicles, and uncoated vesicles – in the oocyte cortex, and studies utilizing electron-dense dyes have confirmed Roth and Porter's hypothesis about the role of coated vesicles in protein uptake (review: Raikhel and Dhadialla, 1992).

Various binding and uptake studies have indicated that Vg is internalized via receptor-mediated endocytosis (RME), and have suggested the presence of specific Vg receptors in the oocytes of *A. aegypti* (Raikhel and Dhadialla, 1992; Sappington *et al.*, 1995), *L. migratoria* (Röhrkasten and Ferenz, 1985; Röhrkasten *et al.*, 1989), *Nauphoeta cinerea* (König and Lanzrein, 1985), *Blattella germanica* (König *et al.*, 1988), *R. prolixus* (Oliveira *et al.*, 1986), *M. sexta* (Osir and Law, 1986), and *H. cecropia* (Kulakosky and Telfer, 1987). However, despite these pioneering studies, the mechanisms of endocytosis have been largely elucidated in other systems, by a combination of animal biochemistry and cell biology, and yeast genetics.

1.3.3.2.2.2. General characteristics of receptor-mediated endocytosis Endocytosis is the cellular process of membrane vesicular transport between the plasma membrane (PM) and cytoplasmic membrane compartments, as well as within the intracellular membrane system. Receptor-mediated endocytosis controls many essential cellular activities, including internalization of essential macromolecules, transmission of neuronal, metabolic and proliferative signals, and defense against invading microorganisms (Goldstein *et al.*, 1985; Bu and Schwartz, 1994).

In RME, an extracellular macromolecule (ligand) is recognized by a specific cell surface receptor. Specific recognition results in high-affinity binding between the ligand and the receptor, which, in turn, triggers endocytosis. The endocytic pathway comprises functionally and physically distinct compartments involved in the internalization of the

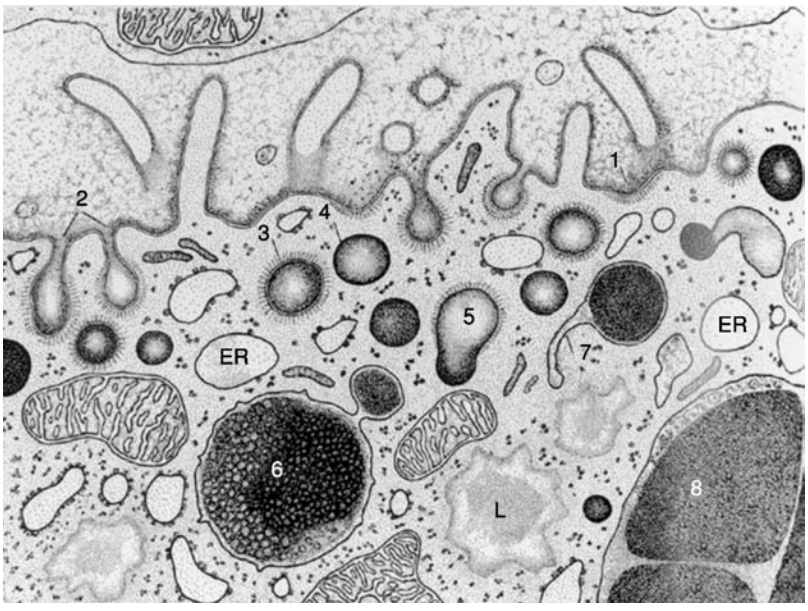


Figure 5 A schematic drawing of protein uptake in the mosquito oocyte: (1) the coated invagination in the plasma membrane of the oocyte; (2) the fully developed coated pit; (3) the coated vesicle; (4) the vesicles after losing their bristles to form dense spheres of similar size; (5) the vesicles fused with other dense spheres; (6) larger droplets coalesced with one another; (7) flattened empty tubular compartment attached to the droplet; (8) larger droplets coalesced to form the large crystalline protein yolk bodies of the oocyte. ER, endoplasmic reticulum; L, lipid droplet. (Reproduced from Roth, T.F., Porter, K.R., **1964**. Yolk protein uptake in the oocytes of the mosquito Aedes aegypti. *J. Cell Biol. 20*, 313–332 by copyright permission of the Rockfeller University Press.)

ligand–receptor complex, the accumulation of ligands, and the recycling of receptors, plasma membrane proteins, and lipids. There are two types of RME, clathrin-dependent and clathrin-independent, defined by the presence or absence of a protein coat on the cytoplasmic side of the vesicular compartments. The main molecular complex of this coat is clathrin (Pearse and Robinson, 1990).

Clathrin-dependent RME, which was first observed in mosquito oocytes, is the major pathway for selective internalization of various macromolecules in insect oocytes through cognate plasma membrane receptors. Goldstein and Brown (1974) were the first to describe RME in mammalian cells and to postulate its general features. In a series of remarkable studies, they characterized the first receptor for RME, the low density lipoprotein (LDL) receptor (LDLR), and identified the pathway for LDL internalization (Goldstein *et al.*, 1985). Amongst the other common macromolecular ligands that animal cells internalize by RME, are transferrin (an iron-binding protein), insulin, and most other peptide hormones (Goldstein *et al.*, 1979, 1985; Mellman, 1996; Simonsen *et al.*, 2001). In insect oocytes, clathrin-dependent RME is responsible for the massive accumulation of Vg and other YPPs (Raikhel and Dhadialla, 1992; Snigirevskaya and Raikhel, 2004). Below, the term RME is used for describing clathrin-dependent RME.

1.3.3.2.2.3. Formation of ligand–receptor complexes, internalization and intracellular fate
Receptor–ligand complexes accumulate in coated invaginations of the PM, called clathrin-coated pits (CCPs) (**Figure 6**, A). These invaginations serve to concentrate cargo proteins into the forming vesicles, and provide a mechanical means to deform a membrane into a vesicular bud. During the progressive assembly of clathrin-coated buds and their maturation, the membrane acquires increasing curvature, until a deeply – invaginated coated pit forms (Takei and Haucke, 2001). When this bud matures, it pinches off giving rise to a clathrin-coated vesicle (CCV).

The clathrin coat has been the subject of extensive biochemical characterization (Pearse and Robinson, 1990; Marsh and McMahon, 1999; Brodsky *et al.*, 2001). Its basic constituent, the clathrin functional unit, is the triskelion consisting of three 180 kDa clathrin heavy chains (CHCs), each complexed with a 30–35 kDa light chain (CLC) (**Figure 6**, B). When triskelions polymerize, they form a curved structure. It has been calculated that each vesicle consists of

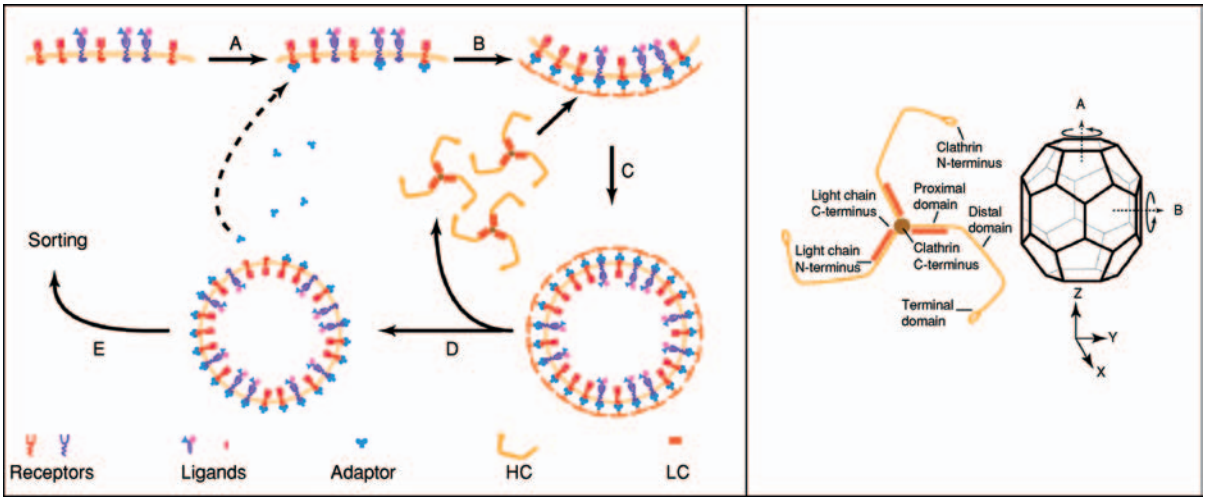


Figure 6 Left panel: early sequential steps of receptor-mediated endocytosis. (A) Receptors are present in the plasma membrane (PM); binding of ligands to their cognate receptors activates recruitment of adaptors that interact with the cytoplasmic domains of receptors. (B) Clathrin triskelions are recruited from the cytoplasmic pool, and assembly of the clathrin lattice on the PM cytoplasmic side is triggered. (C) The clathrin coated vesicle pinches off from the PM and migrates to the cytoplasm. (D) The first step of vesicle uncoating involves detachment of clathrin, which is reutilized for formation of other coated vesicles. In the second step, adaptors are removed and recycled to the PM. (E) The uncoated vesicle fuses with an acceptor compartment, where uncoupling of ligand–receptor complexes and ligand sorting occur. HC, heavy chain of clathrin; LC, light chain of clathrin. (Based on Brodsky, F.M., Chen, C.-Y., Knuehl, C., Towler, M.C., Wakeham, D.E., **2001**. Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu. Rev. Cell Devel. Biol. 17*, 517–586.) Right panel: Schematic diagrams of the clathrin triskelion (left) and the clathrin cage (right). Left: Three clathrin heavy chains (CHC) come together to form a triskelion. The clathrin light chains (CLC) are located at the CHC proximal portions that are being connected at their C-termini. CLCs facilitate triskelion formation. Right: Location of the symmetry axes and the asymmetry unit of the hexagonal clathrin cage. The dashed arrows (A and B) mark the sixfold symmetry axes. The *X*, *Y*, and *Z* axes are defined so that they intersect at the center of the barrel (Smith *et al.*, 1998).

60 triskelions assembled into 20 hexagons and 12 pentagons to achieve the curvature of a CCP (**Figure 6**).

An insect CHC has been characterized in the mosquito *A. aegypti* (Kokoza and Raikhel, 1997; Kokoza *et al.*, 1997). The authors showed that the *chc* gene spans approximately 45 kb, with its coding region divided into seven exons, five of which encode the protein (**Figure 7**). Three distinct mature *chc* transcripts were identified in various mosquito tissues. Two of them are specifically expressed in female germ-line cells encoding isoforms of the CHC polypeptide (M_r of ~191 500) that differ from each other in their NH_2-terminal sequences. The third transcript has a 3′-untranslated region about 1 kb longer than the other variants, and is found only in somatic cells. Tissue-specific 5′-exon splicing and alternative polyadenylation of the primary transcript combine to give rise to these mRNA variants.

The mosquito CHC isoforms are highly conserved, with full-sequence identities of 88% with *D. melanogaster*, 81% with mammal (rat, cow, and human), 71% with *Caenorhabditis elegans*, 58% with *Dictyostelium discoideum*, and 49% with yeast CHC polypeptides. The highest degree of conservation is in the middle portion of the CHC molecule, which includes the linker region and the extended triskelion arm, with decreasing conservation through the N-terminal domain, trimerization domain, and the relatively diverged C-terminal region.

Polyclonal antibodies raised against a bacterially-expressed AaCHC fusion protein recognized one major band of about 180 kDa in whole vitellogenic ovary lysates, while immuno-gold labeling of the AaCHC polypeptide localized it to the coat of CCPs and CCVs of oocytes of vitellogenic follicles (see below). Finally, Northern blot and *in situ* hybridization analyses have suggested that the regulation of *chc* gene expression in the mosquito ovary is complex, and likely involves both developmental and hormonal signals (Kokoza *et al.*, 1997).

After internalization, CCVs rapidly lose their clathrin coats and deliver their contents to early endosomes (EEs), where molecular sorting occurs (**Figure 6**). The assembly and uncoating of CCVs are both energy-dependent processes (Takei and Haucke, 2001). Early endosomes represent a dynamic network of tubules and vesicles dispersed throughout the cytoplasm (Geuze *et al.*, 1983a, 1983b; Tooze and Hollinshead, 1991; Wilson *et al.*, 1991; Gruenberg and Maxfield, 1995; Mukherjee *et al.*, 1997). Their slightly acidic pH (6.0–6.8), which is maintained by an ATP-driven proton pump (Al-Awqati, 1986; Mellman, 1996; Sheff *et al.*, 2002), facilitates the dissociation of many of the ligands contained in them from their receptors.

Free receptors accumulate in the EE's tubular extensions (see below) and are recycled back to the PM (Gruenberg and Maxfield, 1995; Mellman, 1996). The vesicular portions of EEs containing the dissociated ligands traverse to the perinuclear

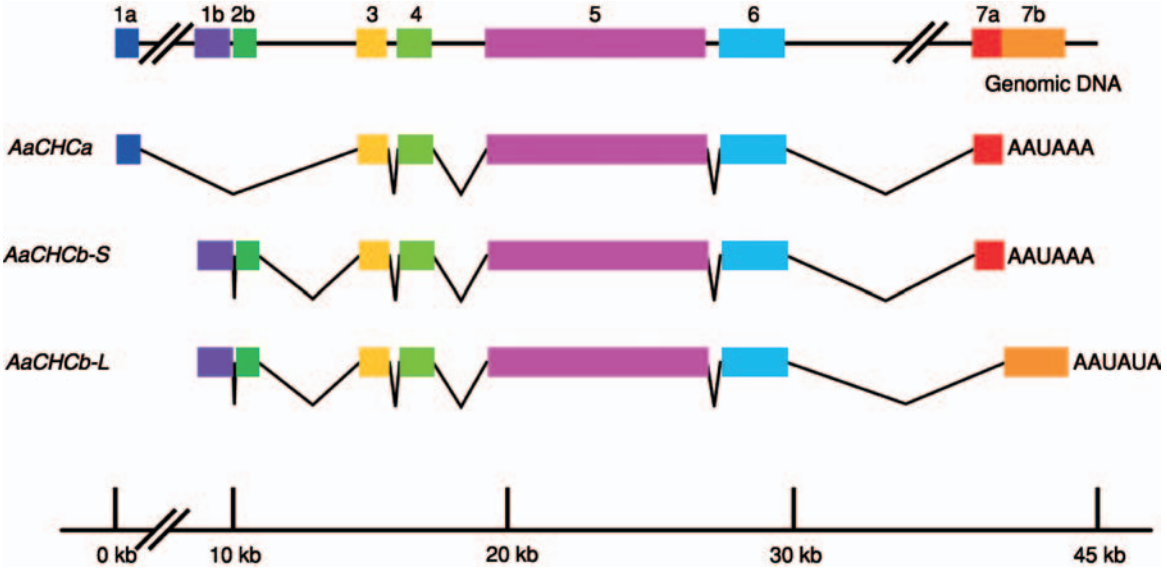


Figure 7 Schematic deduced structure of the *Aedes aegypti chc* gene and its relation to alternatively spliced mRNAs. The *A. aegypti chc* gene is depicted as *boxes* and *lines* representing exons and introns, respectively. Exons are numbered. Three putative transcripts generated from the *A. aegypti chc* gene are shown. Two alternative polyadenylation signals, (AAUAAA) and (AAUAUA), are indicated. (Modified from Kokoza, V.A., Raikhel, A.S., **1997**. Ovarian- and somatic-specific transcripts of the mosquito clathrin heavy chain gene generated by alternative 5′-exon splicing and polyadenylation. *J. Biol. Chem. 272*, 1164–1170, with permission.)

cytoplasm and sort toward late endosomes (LEs) and lysosomes. The LEs accumulate and concentrate internalized contents and partially degraded ligands. From the endosomes, proteins may move to the lysosomes, where they can be degraded by an even lower pH (5.0) and a high concentration of lysosomal enzymes. Some proteins may recycle back to the *trans*-Golgi network or the cell surface for further rounds of transport (Mellman, 1996).

1.3.3.2.2.4. Receptor-mediated endocytotic pathways in insect oocytes In recent years, many details have been elucidated concerning YPP internalization pathways and specific endocytic compartments in insect oocytes (Raikhel and Dhadialla, 1992; Sappington *et al*., 1995; Sappington and Raikhel, 1995; Snigirevskaya *et al*., 1997a, 1997b; Raikhel and Snigirevskaya, 1998; Snigirevskaya and Raikhel, 2004). These studies have revealed that, in general, the sequential steps of RME in oocytes are the same in different insect species. Furthermore, the cellular organelles involved in oocyte RME are similar to those of somatic cells. However, unique features of RME in developing oocytes include an extraordinarily high level of endocytotic organelles in the oocyte cortex, as well as the final routing of internalized YPPs to specialized accumulation organelles, the mature yolk bodies (MYBs), in which YPPs are only minimally processed and are stored until the onset of embryonic development (Telfer *et al*., 1982; Raikhel, 1984; Raikhel and Dhadialla, 1992; Opresko and Wiley, 1987; Giorgi *et al*., 1993a; Raikhel and Snigirevskaya, 1998; Warrier and Subramoniam, 2002; Snigirevskaya and Raikhel, 2004).

1.3.3.2.2.4.1. Receptor-mediated endocytotic pathway of vitellogenin Most studies have been devoted to the internalization of the major YPP, Vg. Studies, at the electron microscopy (EM) level, particularly those that utilized electron dense tracers, have provided the foundation for our understanding of the ultrastructural morphology underlying the RME of YPPs in insect oocytes (see Telfer *et al*., 1982; Raikhel and Dhadialla, 1992).

The purification of Vgs from different insects facilitated the introduction of immunocytochemical methods at the ultrastructural level. A detailed immunocytochemical study was first conducted using developing oocytes of the mosquito *A. aegypti* (Raikhel, 1984). It was shown that Vg binds only along the lower third of oocyte microvilli and between them, suggesting the presence of Vg-specific receptors (VgRs) in these regions of the ooplasm. In adddition, Vg was localized in the organelles of the endocytotic pathway, the CCPs, CCVs, and uncoated vesicular compartments (Raikhel, 1984).

Vitellogenin receptors were purified from the oocytes of *L. migratoria* (Ferenz, 1993) and *A. aegypti* (Sappington *et al*., 1995), and specific anti-VgR antibodies were raised against them. Vitellogenin receptors have been localized subsequently in the oocyte CCPs and CCVs of these insects (Ferenz, 1993; Snigirevskaya *et al*., 1997a). A similar localization of VgR has been described in other oviparous animals, including the chicken *Gallus gallus* and the crab *Scylla serrata* (Schneider, 1992; Warrier and Subramoniam, 2002).

A detailed EM immunocytochemical study of mosquito oocytes has demonstrated the co-localization of Vg and VgR in compartments of the endocytotic pathway (Snigirevskaya *et al*., 1997a). Following initiation of vitellogenesis, the intracellular spaces in the follicular epithelium dilate, facilitating access of macromolecules to the surface of the oocyte. Vitellogenin and its receptor co-localized to specific microdomains at the base of the oocyte microvilli (Snigirevskaya *et al*., 1997a). They were also present in the same compartments of the endocytotic pathway, the CCPs, CCVs, uncoated small vesicular compartments, and EEs (**Figure 8**). Both Vg and VgR were found close to the membrane of these organelles, suggesting that Vg was still bound to its receptors at these stages of internalization (**Figure 8a**).

Uncoated EEs have been observed fusing with one another as well as with larger vesicular compartments, called transitional yolk bodies (TYBs). However, in the TYBs, Vg is localized predominantly in the lumen. Remarkably, numerous tubular compartments are connected to the TYBs, which also are labeled positively with anti-VgR antibodies but not with anti-Vg ones (Snigirevskaya *et al*., 1997a). The differential localization of the ligand and its receptor clearly indicates that the TYB is the organelle for ligand–receptor dissociation, accumulation of Vg, and receptor recycling via tubular compartments (**Figure 8b**). Similar tubular compartments were observed previously in developing vitellogenic insect oocytes (Telfer *et al*., 1982; Raikhel, 1984 Van Antwerpen *et al*., 1993), but the role of these compartments remained unclear, until the direct EM immunolabeling studies with anti-VgR antibodies were conducted with the *A. aegypti* oocytes (Snigirevskaya *et al*., 1997a).

The TYBs are simultaneously in contact with both Vg-containing EEs and VgR-containing tubular compartments. Thus, these data clearly demonstrate that the dissociation of VgR and Vg occurs in the TYBs during coalescence with the EEs, which serve as sorting compartments in somatic cells.

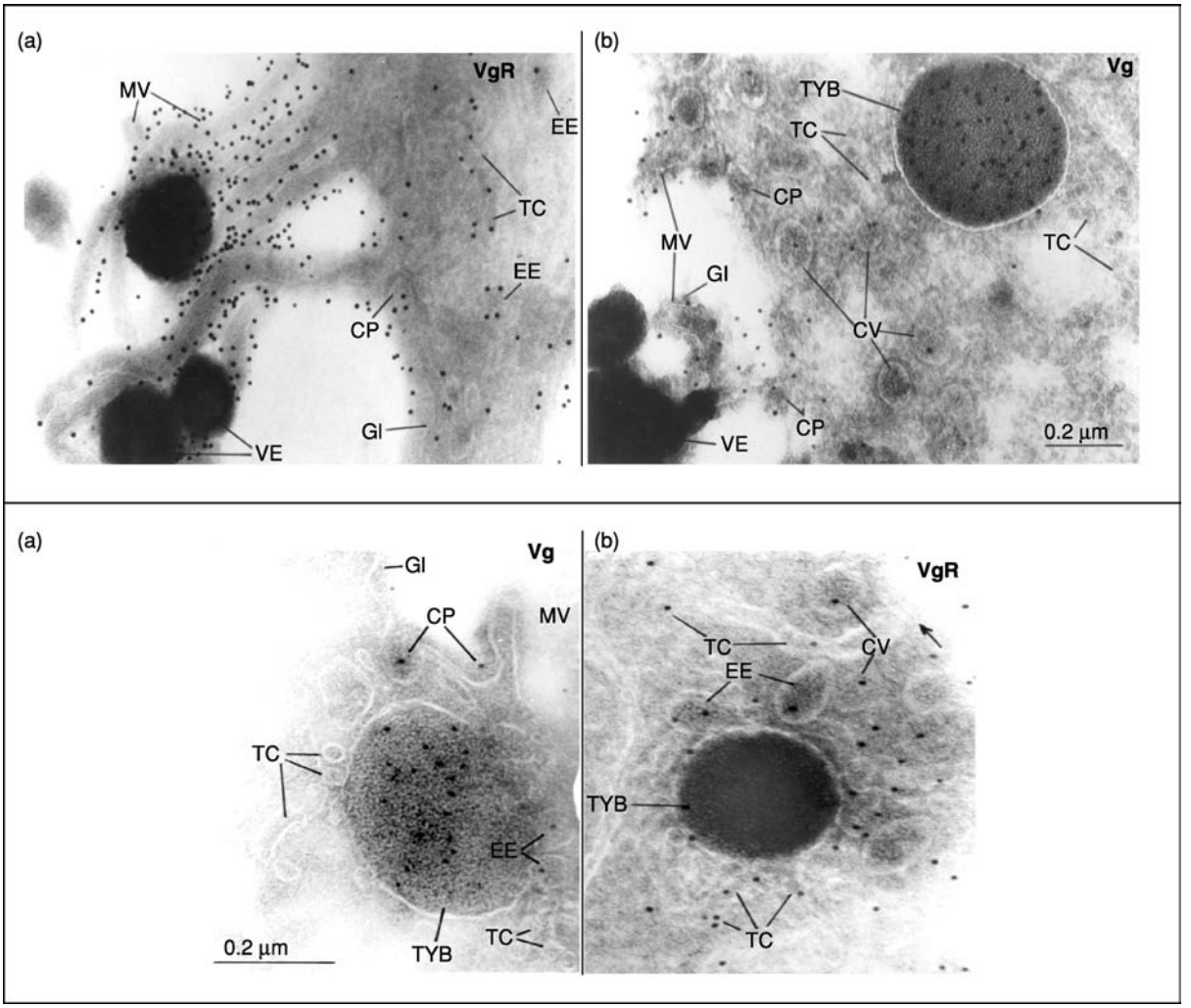


Figure 8 Internalization of vitellogenin (Vg) in the mosquito oocyte visualized through labeling of Vg and vitellogenin receptor (VgR) in ultra-thin frozen sections with 10 nm colloidal gold. Upper panel: The early stages of internalization. (a) Anti-Vg antibodies and protein-gold immunocytochemistry reveals the presence of Vg on the ooplasm membrane at the base of the microvilli (MV), in coated pits (CP), coated vesicles (CV), and the transitional yolk body (TYB), but not in the tubular compartments (TC). (b) Localization of VgR by anti-VgR antibodies and protein-gold immunocytochemistry shows its abundant presence in the oocyte microvillus membrane, CPs, early endosomes (EE), and TCs. Lower panel: The late stages of receptor-mediated endocytosis of vitellogenin in the mosquito oocyte. (a) Anti-Vg antibodies and protein-gold immunocytochemistry reveals the presence of Vg in the lumen of the transitional yolk body (TYB), but not in the tubular compartments (TC). (b) Localization of VgR by anti-VgR antibodies and protein-gold immunocytochemistry shows its abundant presence in TCs. However, in the TYB, VgR label can be found only in TYB membrane. CP, coated pit; EE, early endosome; GL, glycocalyx (surface extracellular coat); MV, microvillus; VE, plaques of the forming vitelline envelope. Scale bar = 0.2 µm. (From Snigirevskaya, E.S., Sappington, T.W., Raikhel, A.S., **1997b**. Internalization and recycling of vitellogenin receptor in the mosquito oocyte. *Cell Tissue Res. 290*, 175–183, with permission.)

Eventually, the TYBs are transformed into mature yolk bodies (MYBs), where vitellin, the crystallized storage form of Vg, is stored (Snigirevskaya *et al*., 1997a, 1997b).

1.3.3.2.2.5. Accumulation of nonvitellogenin yolk proteins Several yolk protein precursors (YPPs) are deposited in developing insect oocytes. In addition, some other female-specific proteins are important for the development of insect oocytes. However, detailed analysis of their internalization is limited.

1.3.3.2.2.5.1. RME of YPs in Drosophila As mentioned earlier, the YPs of cyclorrhaphan Diptera are small (~45 kDa) glycosylated, phosphorylated, and sulfated (Bownes and Pathirana, 2002). Using the *Drosophila* mutant *yolkless* (*yl*), the gene encoding the YP receptor (Yl) has been cloned and characterized (Schonbaum *et al*., 1995; Bownes and Pathirana, 2002). In wild-type vitellogenic oocytes, the receptor is located at the cortex of the egg, and YPs accumulate through the CCPs (Schonbaum *et al*., 2000).

1.3.3.2.2.5.2. Uptake of lipophorin Lipophorin (Lp) serves as a lipid shuttle between the fat body and the developing oocyte, and in some insects, such as lepidopterans and mosquitoes, is sequestered in the yolk (Kawooya and Law 1988; Sun *et al*., 2000; Telfer, 2002; Van Antwerpen *et al*., 2004). Competition experiments with cultured *M. sexta* ovaries indicate that Vg and Lp are internalized by two different receptors as well (Osir and Law, 1986; Kawooya *et al*., 1988). In *H. cecropia*, however, evidence suggests that only a single receptor is involved in endocytosis of these two ligands (Kulakosky and Telfer, 1990). The Lp receptor (LpR) has been cloned and sequenced from both the locust, *L. migratoria* (Dantuma *et al*., 1999) and the mosquito, *A. aegypti* (Cheon *et al*., 2001; Seo *et al*., 2003).

Transient expression studies using vertebrate cell lines demonstrated that the *Locusta* receptor is capable of mediating the endocytic uptake of the *Locusta* high density lipopoprotein (HDLp), lipophorin (Lp). Sun *et al*. (2000) and Cheon *et al*. (2001) have demonstrated direct binding of mosquito Lp to the LpR. It is uncertain, however, how much of the lipid content of *L. migratoria* and *A. aegypti* eggs is derived from receptor-mediated uptake of Lp by the oocyte. A preliminary estimate of the amount of Lp in *Aedes* oocytes indicates that the contribution of internalized Lp to the lipid reserves of the oocyte is limited (Sun *et al*., 2000), suggesting that an additional mechanism exists for lipid delivery to the oocyte. Van Antwerpen *et al*. (1993) conducted a detailed analysis of developing *M. sexta* oocytes with respect to the endocytic pathway for protein incorporation. Three major (lipo)protein components of the mature eggs of this insect, Lp, Vg, and microvitellogenin, were localized along this pathway by immuno-fluorescence and immuno-gold labeling techniques. Labeling of the antigens was initially observed in the extracellular space of the follicle, and the three YPPs have been detected in CCPs and CCVs near the plasma membrane of the oocyte. All three YPPs were observed subsequently in endosomes in the cortex of the oocyte.

The morphology and labeling patterns of the endosomes indicate that this organelle represents the compartment where uncoupling of the receptor from the ligand takes place. In contrast, tubular elements at the surface of the endosome, interpreted to be involved in the recycling of receptors and membranes to the oocyte surface, were not labeled. Strong labeling of Lp, Vg, and microvitellogenin was observed in the developing yolk bodies, the main protein storage compartment of the oocyte. A similar study conducted on *Aedes* oocytes indicates that Vg and Lp are internalized and trafficked together through the same RME pathway (E.S. Snigirevskaya and A.S. Raikhel, unpublished data). Interestingly, both lipoproteins co-localize in the crystalline portion of MYBs.

1.3.3.2.2.5.3. RME of proenzymes serving as YPPs Localization studies of the female-specific YPPs, vitellogenic carboxypeptidase (VCP) and vitellogenic cathepsin B (VCB), in developing mosquito oocytes, have shown that the internalization of these proteins is performed through the same endocytic pathway as Vg. All three YPPs are found on the oocyte microvillus membrane, in CCPs, CCVs, and EEs, and are also co-localized in TYBs (Snigirevskaya *et al*., 1997b). The study, which was based on immuno-gold EM of the developing oocytes of *A. aegypti*, revealed the co-localization of VCP and VCB in most compartments of the RME pathway, from CCPs to TYBs (Snigirevskaya *et al*., 1997a, 1997b). However, VCP and VCB co-localize in the noncrystalline matrix surrounding the compartment where Vn and Lp accumulate (**Figure 9**).

The VCP and VCB proteins are precursors of enzymes participating in yolk degradation during embryogenesis (Raikhel and Dhadialla, 1992; Sappington and Raikhel, 1995). Mature yolk granules can be viewed as a delayed lysosomal compartment particular to oocytes (Wall and Meleka, 1985; Fagotto, 1995). However, in contrast to somatic cells, oocytes do not contain typical lysosomes. In somatic cells, lysosomal enzymes (hydrolases) are delivered from the Golgi complex and initiate the degradation of most ligands in LEs (Gruenberg and Maxfield, 1995; Mellman, 1996; Mukherjee *et al*., 1997). However, in the oocytes of some animals, the presence of enzymes in yolk granules has been described. The enzymes include β-N-acetylglucosaminidase in *Xenopus* (Wall and Meleka, 1985), and serine proteases and cathepsin B and L in *Drosophila* and *Bombyx* (Indrasith *et al*., 1988; Medina *et al*., 1988; Medina and Vallejo, 1989). Giorgi *et al*. (1993b) have suggested that Golgi-derived vesicles deliver hydrolytic enzymes from the Golgi complex to the yolk bodies.

Thus far, the screening of mosquito ovary cDNA libraries has revealed only two RME receptors, VgR and LpR. Ligand-overlay experiments have suggested that VgR is highly specific for Vg, while LpR binds Lp, VCP, and VCB. While binding of Lp occurs by itself, binding of VCP and VCB requires the presence of Lp. Moreover, in co-immunoprecipitation experiments, both VCP and VCB bind Lp (X. Wu, T. Hiraoka, and A.S. Raikhel, unpublished data). This suggests that Lp serves as a carrier for VCP

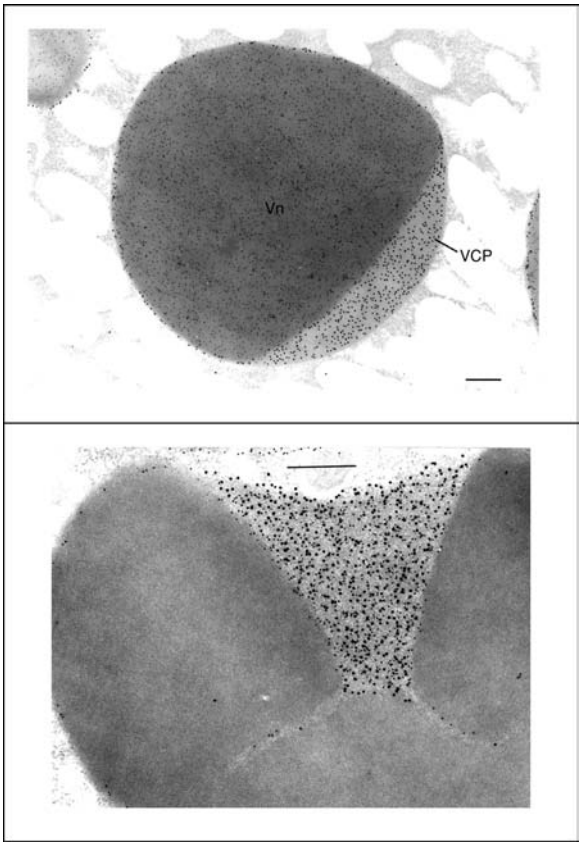


Figure 9 Upper panel: Localization of yolk proteins (vitellin and two processing proteases) in oocyte mature yolk bodies from mosquito females. Double immunolabeling of vitellin (Vn; 10 nm gold particle) and vitellogenic carboxypeptidase (VCP; 15 nm particle) in the mature yolk body of an oocyte from a female mosquito at 24 h post-blood meal. Note the differential localization of these two yolk proteins. Lower panel: Double immunolabeling of vitellogenic carboxypeptidase (VCP; 10 nm gold) and vitellogenic cathepsin B (VCB; 15 nm gold) in the mature yolk body of an oocyte from a female mosquito at 24 h post-blood meal. Note that both these yolk proteins are co-localized in the noncrystalline matrix of the mature yolk body. Bar: 0.5 μm. (Reproduced from Snigirevskaya, E.S., Hays, A.R., Raikhel, A.S., **1997a**. Secretory and internalization pathways of mosquito yolk protein precursors. *Cell Tissue Res. 290*, 129–142, with permission.)

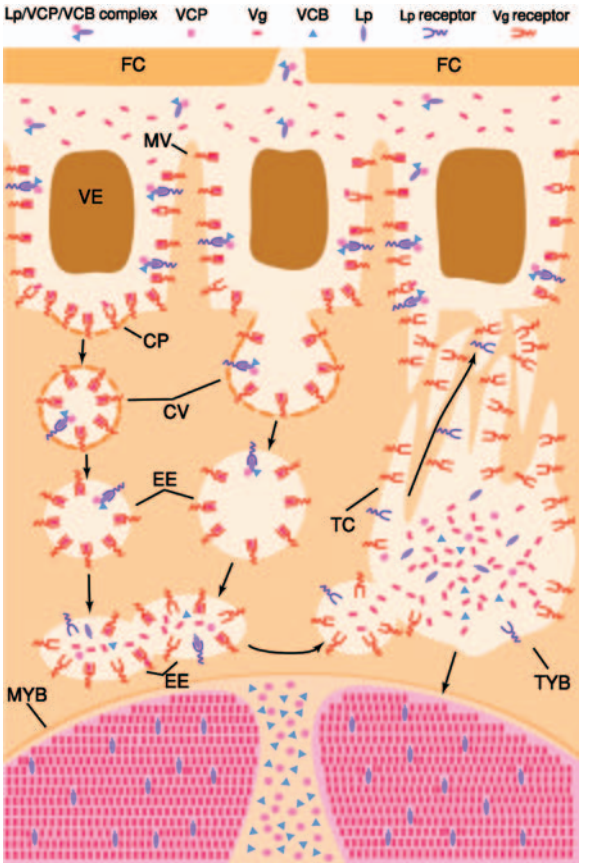


Figure 10 Schematic interpretation of the endocytic pathways and subsequent routing of Vg and VgR, Lp and LpR, VCP and VCB during the internalization of yolk proteins by mosquito oocytes. Ligand–receptor complexes cluster in clathrin-coated pits (CPs) of the vitelline envelope (VE), which invaginate into the cytoplasm and pinch off to form coated vesicles (CVs). VgR binds Vg exclusively, while LpR binds Lp, which presumably transports VCP and VCB via a piggyback mechanism. These complexes are internalized via the same CVs. Upon losing their clathrin coat, the CVs are transformed into early endosomes (EEs), which fuse with one another to form the late endosomes or transitional yolk bodies (TYBs). Early endosomes contain Vg/VgR and Lp/LpR, whereas segregation of the Vg and VgR occurs in the TYBs. Likewise, similar segregation of Lp and LpR is observed. The lumen of the TYB contains four yolk proteins: Vg, Lp, VCP, and VCB. Presumably, Lp, VCP, and VCB are dissociated at this point. VgR and LpR are present only in numerous tubular compartments, which are connected to the TYB and also observed in contact with the ooplasm. The mature yolk bodies (MYBs) contain two parts: the crystalline yolk, composed of modified Vg (vitellin-Vn), and Lp and the non-crystalline matrix, which surrounds Vn/Lp crystals and contains VCP and VCB. (Based on Snigirevskaya *et al.*, 1997a, 1997b; E.S. Snigirevskaya and A.S. Raikhel, unpublished data.)

and VCB, and that internalization of these YPPs most likely occurs via a "piggyback" mechanism. The overall picture that has emerged from studies of RME trafficking of YPPs in the mosquito oocytes is presented in **Figure 10**.

1.3.3.2.3. Oocyte receptors for yolk protein endocytosis

1.3.3.2.3.1. Insect yolk protein receptors Several biochemical studies preceded the cloning and molecular characterization of vitellogenin receptors (VgRs) (reviews: Raikhel and Dhadialla, 1992; Sappington and Raikhel, 1998b). The *L. migratoria* VgR has not been cloned, but its size of 180 kDa was determined on SDS gels (Hafer and Ferenz, 1991). By way of comparison, the mosquito VgR was shown to have a mass of 205 kDa using the ligand-overlay technique (Dhadialla *et al.*, 1992; Sappington and Raikhel, 1995).

As of the writing of this chapter, there are only three insects from which cloned VgR sequences have been reported, the yellow fever mosquito, *A. aegypti* (Sappington *et al*., 1996), the fire ant, *Solenopsis invicta* (Chen *et al*., 2004), and the cockroach, *P. americana* (Acc. no. BAC02725). A database search of the recently published genome sequence of the malaria mosquito, *Anopheles gambiae* (Holt *et al*., 2002), yielded a predicted amino acid sequence (Acc. no. EAA06264) with 54% identity to the *A. aegypti* VgR sequence. This will be referred to here as the *A. gambiae* VgR (Sappington and Raikhel, 2004).

There is no evidence for Vg production or Vg genes in cyclorrhaphan flies like *Drosophila*, which instead utilize smaller unrelated YPs as their major yolk proteins (Bownes and Pathirana, 2002). The YP receptor in *D. melanogaster* was identified and cloned by taking advantage of the *yolkless* (*yl*) mutation (Schonbaum *et al*., 1995), and is referred to as the yolkless protein, Yl. Despite their unrelated ligands, the Yl shares high sequence identity with insect VgRs (Sappington *et al*., 1996; Sappington and Raikhel, 2004), and polyclonal antibodies raised to *A. aegypti* VgR recognize the *D. melanogaster* Yl (Richard *et al*., 2001) indicating similar tertiary structures.

Dantuma *et al*. (1999) cloned the *L. migratoria* Lp receptor (LpR) and determined that it is a member of the LDL receptor (LDLR) family. The locust LpR is expressed primarily in the fat body, but it is also present in the ovary. The cDNA for a similar lipoprotein receptor (LpR), which is likely responsible for the uptake of Lp by *A. aegypti* oocytes (Sun *et al*., 2000), has been cloned by Cheon *et al*. (2001). Two splice variants of *LpR* mRNA have been identified that give rise to AaLpRfb and AaLpRov, which are specific for the fat body and the ovary, respectively (Seo *et al*., 2003). In addition to recognizing Yl, anti-*Aedes* VgR polyclonal antibodies recognize an abundant 80 kDa protein in *D. melanogaster* ovaries, which possibly represents an ovarian LpR, in addition to Yl (Richard *et al*., 2001). Genes encoding putative LpRs also have been identified in the *Drosophila* genome (Culi and Mann, 2003). Thus, two distinct receptors appear to exist for YPs and Lp in this species.

1.3.3.2.3.2. The superfamily of low-density lipoprotein receptors The insect VgRs, Yl, and LpRs are all members of the low-density lipoprotein receptor (LDLR) superfamily, named after the LDLR first described in humans (Yamamoto *et al*., 1984). LDLR-superfamily members are characterized by various arrangements of five structural domains (**Figure 11**): (1) *class A complement-type imperfect repeats*, often referred to as ligand-binding repeats (LRs), each containing six cysteine residues that form an invariant pattern of disulfide bonds (Bieri *et al*., 1995a, 1955b); (2) *class B EGF-type imperfect repeats*, also containing six cysteine residues, which, however, form a pattern of disulfide bonding different than that of the cysteines in the class A repeats (Campbell and Bork, 1993); (3) *YWXD imperfect repeats*, invariably occurring in groups of six, which fold to form a compact 6-bladed β-propeller structure (Springer, 1998; Jeon *et al*., 2001) that is involved in acid dissociation of the receptor and its ligand in the endosome (Innerarity, 2002) – this domain is often considered part of a larger EGF precursor homology domain, which includes the class B repeats (Nimpf and Schneider, 1998; Hussain *et al*., 1999); (4) a *single-pass transmembrane domain* near the C-terminus; and (5) a *cytoplasmic tail* that includes one or more tyrosine or dileucine-based internalization signals. In addition, there is often an extracellular *O*-linked sugar region proximal to the membrane, and in some cases, the presence or absence of this domain is determined by differential splicing (e.g., Bujo *et al*., 1995). Besides the presence of these domains, Hussain *et al*. (1999) list other characteristics of LDLR-superfamily members, including cell-surface expression, a requirement of Ca^{2+} for ligand binding, and internalization of ligands by receptor-mediated endocytosis.

One of the most striking structural characteristics noticed when comparing LDLR-superfamily members, is the clustering of class A modules into 1–4 distinct ligand-binding domains, with a characteristic number of modules per domain (**Figure 11**). Complete VgR sequences are known from five species of vertebrates (Bujo *et al*., 1994; Okabayashi *et al*., 1996; Davail *et al*., 1998; Li *et al*., 2003; Hiramatsu *et al*., 2004), and are characterized by a single ligand-binding domain containing eight class A modules. Similarly, the mosquito ovarian LpR (AaLRov) has eight class A modules in a single cluster (Cheon *et al*., 2001). Alternatively, the fat body-specific LpR isoform (AaLpRfb) consists of seven complement-type, cysteine-rich repeats in its putative ligand-binding domain, which are identical to the second through eighth repeats of AaLpRov. The *AaLpRov* transcripts are present exclusively in ovarian germ-line cells, nurse cells, and oocytes, while the *AaLpRfb* transcripts are fat body-specific (Seo *et al*., 2003). The differences between these two isoforms suggests that they internalize Lps with different apo-Lp compositions.

Finally, insect VgRs/Yl have two clusters of class A modules (**Figure 11**). The first cluster contains five modules in all except for the *S. invicta* VgR, which only has four. The second cluster contains

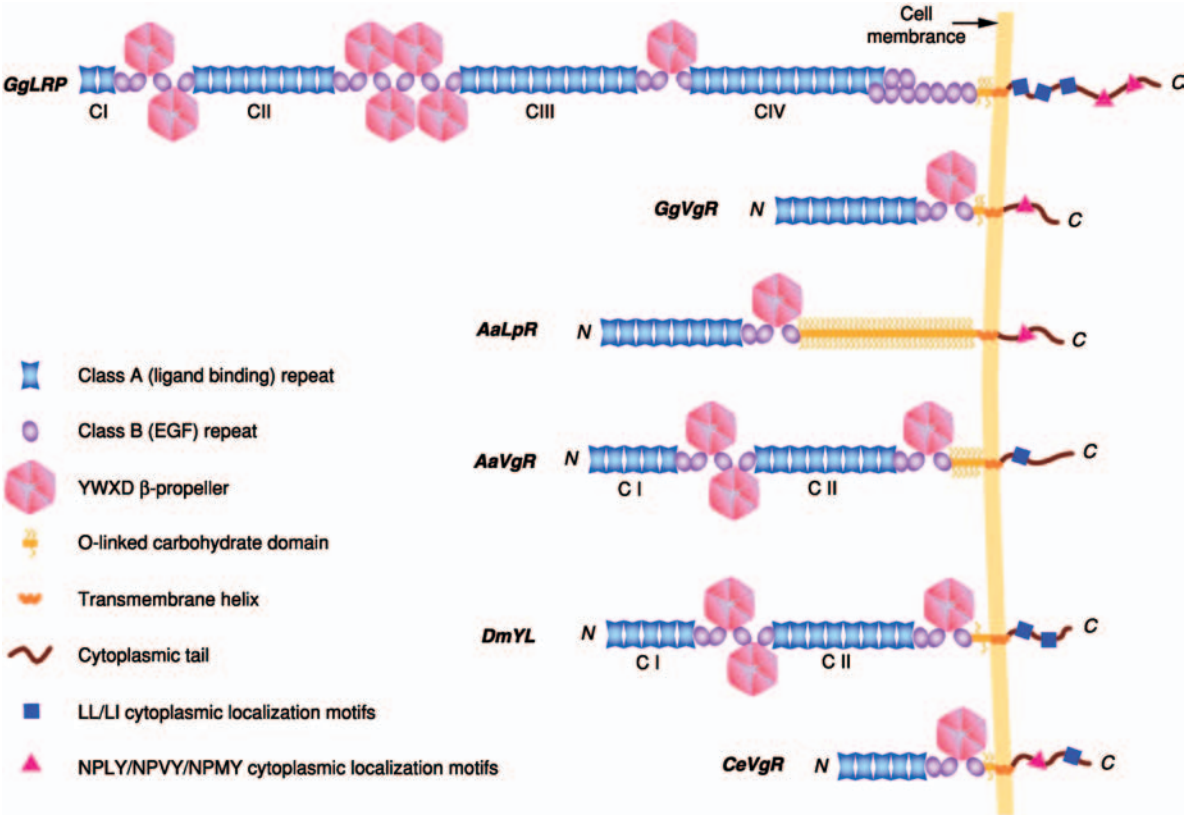


Figure 11 Schematic illustration of receptors of the LDLR superfamily, which are known to internalize yolk protein precursors into oocytes, emphasizing the arrangement of various modular domains. The C-terminus of each receptor is located on the cytoplasmic side of the cell membrane, while the N-terminus is extracellular. CI, first cluster of class A (ligand-binding) repeats in multi-cluster receptors; CII, second cluster, etc. The relative locations of tyrosine-based (NPXY) or dileucine-based (LL or LI) internalization signals in the cytoplasmic tail are also indicated. *GgLRP*, chicken (*Gallus gallus*) LDLR-related protein; *GgVgR*, chicken (*G. gallus*) vitellogenin receptor; *AaLPR*, mosquito (*Aedes aegypti*) lipophorin receptor; *AaVgR*, mosquito (*A. aegypti*) vitellogenin receptor; *DmYl*, fruit fly (*Drosophila melanogaster*) yolkless protein (or YP receptor); *CeVgR*, nematode (*Caenorhabditis elegans*) vitellogenin receptor. (Modified from Sappington, T.W., Raikhel, A.S., **2004**. Insect vitellogenin/yolk protein receptors. In: Raikhel, A.S., Sappington, T.W. (Eds.), Progress in Vitellogenesis, vol. XII, Part B. Science Publishers, Inc., Enfield, NH, pp. 229–264.)

either eight (*A. aegypti* VgR and *D. melanogaster* Yl) or seven repeats (*P. americana* and *A. gambiae* VgRs). A search of the *D. melanogaster* genome (Adams *et al.*, 2000) revealed the presence of seven LDLR superfamily members (Culi and Mann, 2003), including homologs of the very large LDLR-Related Protein-2 receptors of vertebrates (Herz and Bock, 2002). The role of these large receptors in insects is unknown. For a more detailed analysis of insect RME receptors, the characteristics of their domains and their functional relevance, the reader is referred to a recent review by Sappington and Raikhel (2004).

1.3.3.2.4. Concluding remarks In addition to the physiological and functional importance of yolk accumulation in the oocyte to any reproducing insect, the processes and proteins involved are fundamental to many aspects of cell biology in animals in general. For example, RME of macromolecules is a ubiquitous process of eukaryotic cells (Goldstein *et al.*, 1979; Schwartz, 1995). Insect Vgs are related to the Vgs of vertebrates and nematodes (Chen *et al.*, 1997), and belong to a yet larger superfamily of proteins called large lipid transfer proteins (LLTP) (Babin *et al.*, 1999). The apolipoprotein component of vertebrate LDL, a molecule involved in cholesterol homeostasis (Brown and Goldstein, 1986), is an LLTP (Byrne *et al.*, 1989; Chen *et al.*, 1997). Insect VgRs, Yl, and LpRs are related to vertebrate and nematode VgRs, and all belong to the larger LDLR superfamily, which bind a variety of ligands including LLTPs (Sappington and Raikhel, 1998a, 1998b, 2004; Babin *et al.*, 1999). The YPs of *Drosophila* are related to lipoprotein lipases (Bownes *et al.*, 1988b), a ligand of certain vertebrate LDLR superfamily receptors (Beisiegel, 1996). Thus, the evolutionary connections among these proteins in widely divergent animal taxa are pervasive. The importance of this cannot be overemphasized, because the commonalities in these systems

make studies on the mechanisms of insect yolk accumulation relevant to mechanisms of cholesterol homeostasis and LDLR-based genetic diseases in humans (Gliemann, 1998; Herz and Farese, 1999; Hussain *et al*., 1999; Willnow, 1999; Nykjaer and Willnow, 2002; Strickland *et al*., 2002), and *vice versa*. Thus, those working in these normally disjunct fields of research have much to offer and learn from one another (Sappington and Raikhel 1998b, 2004; Sappington *et al*., 2002).

1.3.3.3. The Transition from Vitellogenesis to Choriogenesis

At the end of vitellogenesis, the cells of the follicular epithelium lose their patency, and this results in a cessation of yolk protein transport to the oocyte surface (Wang and Telfer, 1996). Concurrently, gene expression in the cells of the follicular epithelium switches from yolk protein gene transcription to the activation of eggshell or chorion protein-encoding genes (Kafatos *et al*., 1977). Prior to choriogenesis, the nurse cells also empty their cytoplasmic contents into the oocyte and undergo apoptosis (Mahajan-Niklos and Cooley, 1994).

During the vitellogenic stages that precede the onset of choriogenesis, other important events occur in follicles, including the synthesis of vitelline membrane proteins and ovarian (maternal) ecdysteroids by the cells of the follicular epithelium (Kadono-Okuda *et al*., 1994; Kendirgi *et al*., 2002), the reinitiation of meiosis in the oocytes (Lanot *et al*., 1990), and morphogenetic movements of the follicular epithelium prior to their commitment to chorion protein gene expression (Spradling, 1993; Montell, 2001).

1.3.3.3.1. Diptera As is the case for the transition from previtellogenesis to vitellogenesis, most of the information regarding the transition from vitellogenesis to choriogenesis comes from the two major insect models, the fruit fly *D. melanogaster*, and the mosquito *A. aegypti*.

1.3.3.3.1.1. **Drosophila melanogaster** Particular elements of the process of transition from vitellogenesis to choriogenesis, such as the patterning and movements of the follicular epithelium and the nurse cell dumping process, have been analyzed in detail by genetic methods in *Drosophila*. These processes will be discussed briefly in Sections 1.3.3.6 and 1.3.3.7, respectively, and are also reviewed in more detail in **Chapter 1.2**.

No detailed *in vitro* culture experiments of *Drosophila* ovarian follicles have been carried out to determine whether the transition from vitellogenesis to choriogenesis occurs as a follicle-autonomous process or whether it requires the presence of extra-ovarian factors (see also discussion in Section 1.3.3.3.2.1). Detailed measurements of ecdysteroid levels with respect to follicle development have also not been carried out in order to deduce whether the transition to choriogenesis is accompanied by a decline of ecdysteroid production in the cells of the follicular epithelium (see also discussion for *A. aegypti* and *B. mori* in Sections 1.3.3.3.1.2 and 1.3.3.3.2.1, respectively). Furthermore, genetic analysis has yet to reveal the components of a "master" regulatory pathway that governs the transition from vitellogenesis to choriogenesis in *Drosophila*. Therefore, the mechanism by which the cells of the follicular epithelium differentiate from a patent state that is involved in the synthesis of yolk proteins and allows free passage of hemolymph yolk proteins to the oocyte surface, to one that becomes specialized for chorion protein production, remains unknown in *Drosophila*.

1.3.3.3.1.2. **Aedes aegypti** ***and anautogenous flies*** In *A. aegypti*, the transition from vitellogenesis to choriogenesis is accompanied by a decline in ecdysteroid levels (Hagedorn, 1985). A decline in the abundance of the mRNAs of nuclear receptors involved in the ecdysone regulatory pathway, i.e., *AaEcR*, *AaUSP*, *AaE74B*, *AaE75A/B/C*, *AHR3*, also occurs in the ovary at the time of termination of vitellogenesis (Cho *et al*., 1995; Pierceall *et al*., 1999; Kapitskaya *et al*., 2000; Wang *et al*., 2000; Sun *et al*., 2002). Also, the levels of the nuclear receptor AaHNF4b are down-regulated at this transition (Kapitskaya *et al*., 2000). By contrast, the levels of *AaE74A* mRNA increase at the end of vitellogenesis and during choriogenesis (Sun *et al*., 2002). The changes in the expression levels of the above factors suggest their involvement in the regulation of the transition from vitellogenesis to choriogenesis. However, as has been discussed in Section 1.3.3.1.1.2, it is not clear whether the expression levels of the above nuclear receptors in ovarian tissue are under control of the ecdysone regulatory pathway.

In the mosquito, a peptide hormone involved in the termination of vitellogenesis, trypsin-modulating oostatic factor (TMOF), is produced during vitellogenesis by the cells of the follicular epithelium (Borovsky *et al*., 1990, 1994b). The target of TMOF is the gut, where it inhibits biosynthesis of trypsin and stops the breakdown of blood proteins that are essential for vitellogenesis (Borovsky *et al*., 1990, 1994a, 1994b) (see also **Chapters 1.5** and **3.9**). Thus, the action of TMOF blocks the availability of nutrients necessary for

continuation of vitellogenesis. Because TMOF is produced by the cells of the follicular epithelium, a model emerges, in which ovarian follicles produce TMOF upon reaching a certain size, in order to prevent further growth and initiate post-vitellogenic events (De Loof *et al*., 1995).

A functional analog of TMOF (Neb-TMOF) has also been isolated from *Neobellieria* (*Sarcophaga*) *bullata*, a fly that requires a protein meal to initiate vitellogenesis (anautogenous flies; Huybrechts and De Loof, 1977). Neb-TMOF is produced by vitellogenic follicles but, in this case, the oocyte rather than the cells of the follicular epithelium is the major source of the peptide (Bylemans *et al*., 1996). The effects of Neb-TMOF include inhibition of trypsin biosynthesis in the gut and concomitant lowering of yolk protein concentration in the hemolymph (Bylemans *et al*., 1994). A second oostatic factor or folliculostatin of *Neobellieria*, Neb-colloostatin, however, does not inhibit the synthesis of trypsin-like molecules in the gut and does not terminate viellogenesis in vitellogenic follicles. Instead, the peptide prevents previtellogenic follicles from entering vitellogenesis, and may be involved in the feedback mechanism that blocks vitellogenic growth of the penultimate oocyte before completion of vitellogenesis in the terminal follicle (Bylemans *et al*., 1995).

In the female blowfly, *Phormia regina*, ovarian ecdysteroidogenesis is regulated by antagonistic activities of cAMP (positive regulation) and cGMP (negative regulation) second messengers (Manière *et al*., 2000, 2003). Calcium signaling is also involved, since calcium ions inhibit steroidogenesis, presumably by activating a calmodulin-sensitive (type I) phosphodiesterase (Manière *et al*., 2002). Cyclic GMP levels in the blowfly ovary reach their maximal levels at the end of vitellogenesis, in close association with a decrease in ecdysteroidogenesis. Thus, cGMP may signal the end of vitellogenesis through inhibition of ovarian ecdysone production and concomitant inhibition of ecdysone-regulated yolk protein production. The regulation of cGMP levels does not seem to be dependent on extra-follicular factors but may involve paracrine or autocrine factors, possibly nitric oxide (Manière *et al*., 2003).

1.3.3.3.2. Lepidoptera Information regarding the regulation of the transition from vitellogenesis to choriogenesis in lepidopteran insects has been derived almost exclusively from one species, the domesticated silkmoth, *B. mori*.

***1.3.3.3.2.1.* Bombyx mori** The developing ovariole of the silkmoth, *B. mori*, represents an excellent system for the study of changes in gene expression during follicle development (Swevers and Iatrou, 2003). Because of the asynchronous nature of oogenesis (**Figure 12**), follicles at all different stages of development are simultaneously present and arranged in a linear array in each of the animal's 8 ovarioles (Bock *et al*., 1986; Swevers and Iatrou, 1992), with each follicle differing from its immediate neighbor by 2–2.5 h of developmental distance. This feature allows the isolation of all stages of oogenesis by dissection of a single female pharate adult.

Because the transition between vitellogenic and choriogenic follicles is easily visible by eye, a

Figure 12 Structure of the developing ovary of the silkmoth, *Bombyx mori* at different stages of development. Upper panel: Development of the ovary of the silkmoth, *B. mori*, in spinning larvae, pupae and pharate adults. In spinning larvae (SL) and pupae until 12 h after larval–pupal ecdysis (P0 and P0 + 12 h), the ovarioles are still enclosed within a protective capsule. When ecdysteroid titers rise in the hemolymph, the capsule ruptures and the ovarioles emerge in the abdominal cavity (P1, P1 + 12 h). Two to three days after ecdysis, vitellogenesis is initiated (P2 and P3) while choriogenesis starts 5 days after ecdysis (P5). During the second half of pharate adult development, choriogenic follicles accumulate in the ovarioles and ovulation ensues (P7). SL, spinning larvae; Pn (+12 h), pupae or pharate adults, n days (+12 h) after larval pupal ecdysis. Lower panel: Structure of a silkmoth ovariole, 6–7 days after larval–pupal ecdysis. Indicated are the various stages of vitellogenesis (−1 to −nn) and choriogenesis (+1 to +nn). In this numbering system, vitellogenesis is initiated at −60, while choriogenesis starts at +1. (Lower panel: Reprinted with permission from Swevers, L., Iatrou, K., **2003**. The ecdysone regulatory cascade and ovarian development is lepidopteran insects: insights from the silkmoth paradigm. *Insect Biochem. Mol. Biol.* 33, 1285–1297; © Elsevier.)

numbering system has been devised to stage the development of follicles (Kafatos *et al*., 1977). In this numbering system, the first choriogenic follicle acquires the number +1 and successive later stages of choriogenic development (differing by 2.5 h of developmental distance) are given correspondingly higher positive numbers; vitellogenic follicles, alternatively, are numbered with negative numbers, with follicle −1 representing the last stage of vitellogenesis (**Figure 12**).

The developing ovariole is especially suitable for the study of program of follicular cell differentiation from middle vitellogenesis to choriogenesis (stages −30 to −35 and later), because at these stages individual follicles have reached a size sufficiently large to allow easy manipulation for physiological, biochemical, and gene expression studies. Thus, Northern blot analysis has been used to elucidate the changes in the expression patterns of transcription factors during the transition from vitellogenesis to choriogenesis, and correlate such expression patterns with those of structural genes, e.g., genes encoding yolk, vitelline membrane, and chorion proteins (Swevers and Iatrou, 1999, 2003). In many cases, the expression analysis of the mRNA has been coupled with the detection of the corresponding proteins in the follicles by Western blot analysis and immunocytochemistry.

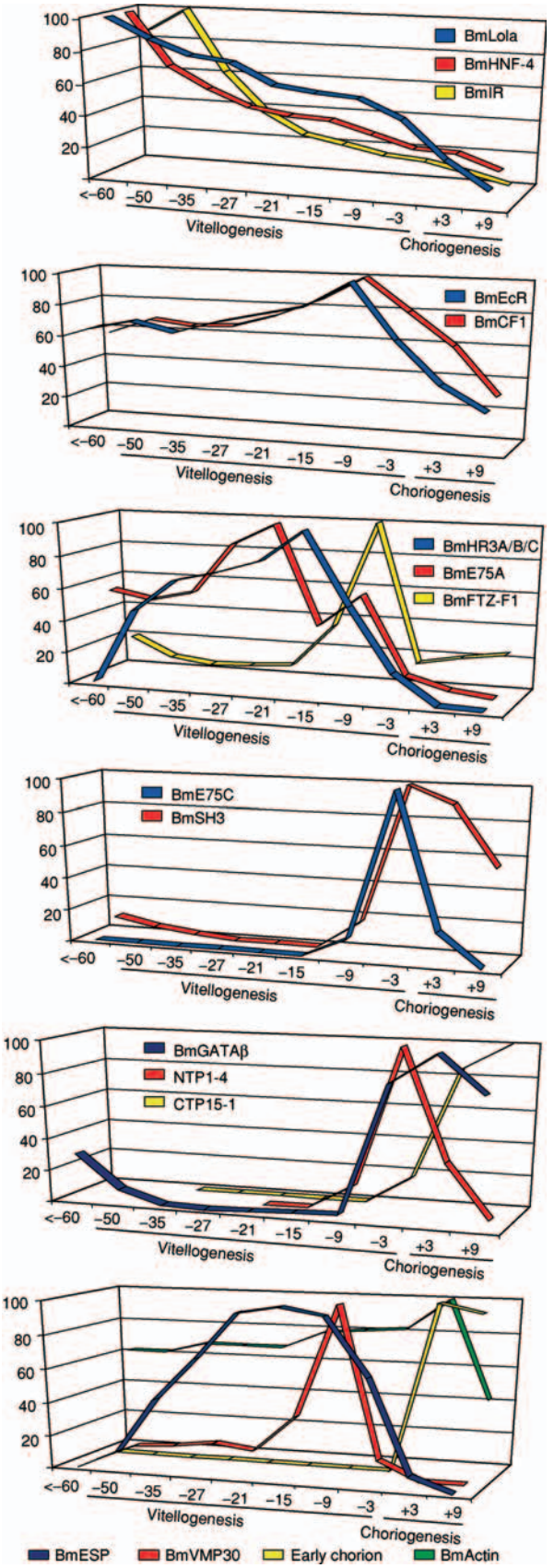
1.3.3.3.2.1.1. In vitro *culture experiments* Mid to late vitellogenic follicles (stages from approximately −35 onwards) are capable of developing in organ culture in a chemically defined medium, completing vitellogenesis and, also, entering and completing the choriogenic program autonomously (Swevers and Iatrou, 1992). Previously, it was also shown that the program of chorion gene expression in the American silkmoth, *Antheraea polyphemus* is also follicle-autonomous (Paul and Kafatos, 1975)

However, it was also observed in *Bombyx* that only a limited number of middle vitellogenic follicles (stages −30/−35 and later) have the capacity to develop autonomously *in vitro* (Swevers and Iatrou, 1992). This finding suggested that follicles of earlier vitellogenic stages require the presence of an extraovarian (hemolymph) factor(s), which triggers the activation of a regulatory cascade allowing the follicles to complete vitellogenesis, and enter and complete choriogenesis autonomously. According to the proposed model, follicles at stages −35/−30 and beyond (at the time of dissection), have acquired the competence to respond to a circulating signal (e.g., a hormone present in the hemolymph) that allows them to develop further autonomously. The term competence implied the stage-specific appearance in the follicles (probably the cells of the follicular epithelium) of a receptor that allows the binding of the circulating signal and the triggering of a relevant regulatory cascade, which establishes the autonomy of the differentiation program. Thus, follicles lacking this capacity (at stages earlier than −35) are unable to respond to the putative signal and cannot proceed autonomously through the execution of the autonomous part of the differentiation program (Swevers and Iatrou, 1992).

To elucidate the nature of the postulated extraovarian factor(s), two approaches have been considered useful: (1) to culture ovarioles in the presence of hemolymph (and, eventually, purified hemolymph factors) or known hormones, and compare the developmental potential of follicles in supplemented versus nonsupplemented culture media; and (2) to search for genes encoding putative receptors and expressed in follicular cells at or around stages −30/−35 but not at earlier stages, because genes implicated in the transduction of the "autonomy signal" would be expected to be differentially expressed during these stages.

Following upon these two approaches, it has been demonstrated that the hormone 20E does not play a role in the induction of the choriogenic program in the follicular cells at mid vitellogenesis. Thus, addition of 20E to ovariole cultures did not change the capacity of vitellogenic follicles to develop autonomously toward subsequent stages (Swevers and Iatrou, 1992). Furthermore, it was determined that the components of the ecdysone receptor heterodimer, BmEcR and BmUSP, as well as the known downstream signaling components in the ecdysone regulatory pathway, are not expressed differentially in the cells of the follicular epithelium with respect to the stages of establishment of follicle autonomy (**Figure 13**) (Swevers *et al*., 1995; Swevers and Iatrou, 2003).

Subsequent studies to address the issue of the establishment of developmental autonomy in the ovarian follicles have focused on bombyxins, a class of insulin-like peptides that have been isolated from *B. mori* brain extracts based on their prothoracicotropic activity (capacity to activate the prothoracic glands) in the moth *Samia cynthia ricini* (Nagasawa *et al*., 1984; Jhoti *et al*., 1987). The functions of bombyxins in *Bombyx* relate to the regulation of glucose metabolism and growth control (Satake *et al*., 1997; Masumura *et al*., 2000). High titers of bombyxins are also found in the hemolymph of female pharate adults suggesting an (as yet undefined) role in the regulation of female reproduction, possibly ovarian development (Saegusa *et al*., 1992). Bombyxin-binding activity is detected in ovarian follicle membrane extracts,



indicating that ovarian tissue is a direct target tissue of bombyxin peptides (Fullbright *et al*., 1997). Furthermore, the expression of bombyxin mRNAs in ovarian tissue (Iwami *et al*., 1996) also indicates a possible autocrine or paracrine role for bombyxins during follicle development. A gene encoding a putative receptor for bombyxins, the *Bombyx* insulin receptor-like (BmIRL) protein, was cloned (**Figure 14**; I. Lindström-Dinnètz and K.I., unpublished data), and the expression pattern of its mRNA during follicle development determined (Swevers

Figure 13 Schematic overview of the expression of the mRNAs of regulatory factors during follicular development in *Bombyx mori* as detected by Northern blot and quantified by Phosphorimager analysis. Expression levels for each gene are plotted relative to the highest signal (100%). Genes were grouped to show similarity in expression levels (first, second, fourth, and fifth panels), to illustrate cross-regulation (third panel) or to indicate broad developmental periods (structural genes in sixth panel). First panel: Genes whose expression levels decline gradually as oogenesis progresses: BmHNF-4, BmIRL, and BmLola. BmLola is the homolog of the *Drosophila* transcription factor Lola that is characterized by an N-terminal BTB-domain and C-terminal C_2H_2 zinc fingers (Giniger *et al*., 1994). Second panel: Expression of the mRNAs of the components of the ecdysone receptor heterodimer, BmEcR and BmUSP. Both mRNAs are present at similar levels throughout most of vitellogenesis, peak at the end of vitellogenesis, and decline during choriogenesis. Third panel: Expression of the nuclear receptors BmHR3A, BmE75A, and BmFTZ-F1. Note that BmFTZ-F1 becomes induced at maximal values of BmHR3A, concomitantly with a drop in expression levels of BmE75A. Fourth panel: Induction of the mRNAs of the nuclear receptor BmE75C and the adaptor protein BmSH3 at the end of vitellogenesis. The two factors interact in yeast two-hybrid assays and may form a functional complex in the follicular cells. Fifth panel: Induction of the mRNAs of BmGATAβ and two putative interacting factors (NTP 1–4 and CTP 15–1; Glushek, 2001) in the follicular epithelium at the end of vitellogenesis. Sixth panel: Expression of structural genes and developmental markers. ESP is the yolk protein produced by the follicular cells and is a marker of vitellogenesis, BmVMP30 is a vitelline membrane protein expressed during mid-late vitellogenesis, and early chorion genes are induced at the beginning of choriogenesis. BmActin corresponds to the cytoplasmic actin gene; note that its expression shows a peak at the beginning of choriogenesis followed by a sharp decline. Numbers at the bottom of each graph indicate the stages of oogenesis with respect to the start of choriogenesis (+1). The expression levels that are indicated are obtained from pools of 6 follicles (+9, +3, −3, −9, −15, −21, −27), 10 follicles (−35), 20 follicles (−50), or represent the remainder of follicles earlier than −60 (<−60). The numbers indicate the follicle that is the midpoint of each series of 6 follicles (for instance, +9 = +7/+12), 10 follicles (−35 = −31/−40), or 20 follicles (−50 = −41/−60). ''<−60'' represents a large number (>50) of early follicles. The broad developmental periods of vitellogenesis and choriogenesis are also indicated. (Reprinted with permission from Swevers, L., Iatrou, K., **2003**. The ecdysone regulatory cascade and ovarian development in lepidopteran insects: insights from the silkmoth paradigm. *Insect Biochem. Mol. Biol. 33*, 1285–1297; © Elsevier.)

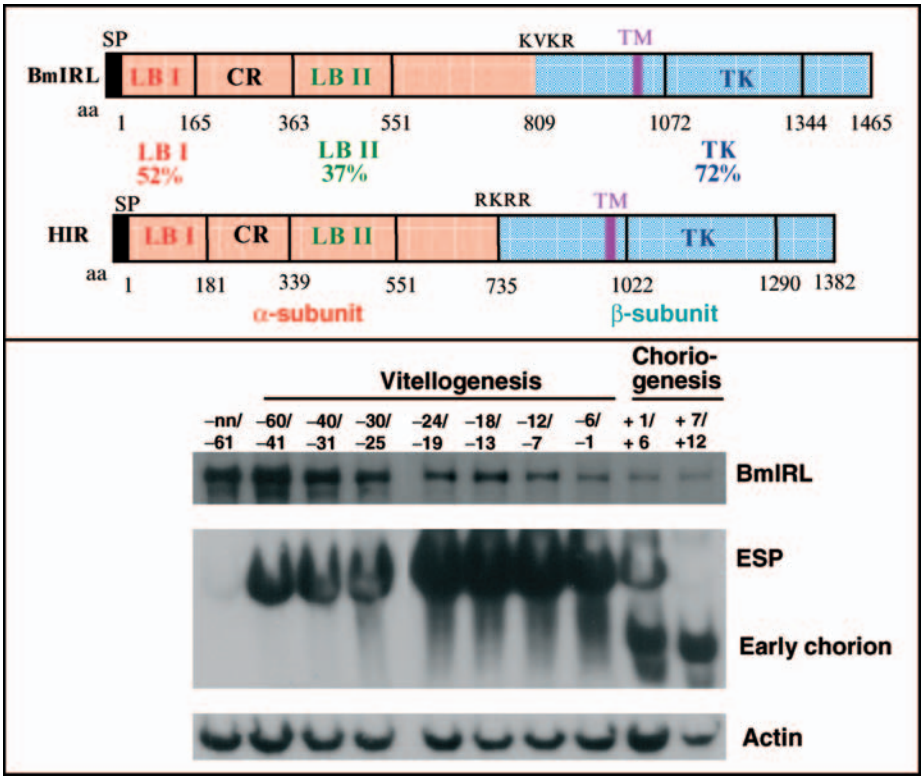


Figure 14 Structure and expression pattern of the *Bombyx* insulin-like receptor (BmIRL; GenBank accession number AF025542). Upper panel: Schematic drawing of the BmIRL protein and comparison with the human insulin receptor (HIR). Indicated are the signal peptide (SP), the ligand-binding domains I and II (LB I and II), the cysteine-rich region (CR) of the α-subunit, the documented cleavage site (RKRR) for processing of HIR into α- and β-subunits, the transmembrane region (TM), and the tyrosine kinase (TK) domain of the β-subunit. Note that the proteolytic processing at the putative cleavage site (KVKR) of BmIRL has yet to be demonstrated. Percentages indicate % identity of selected domains with the corresponding domains of the human insulin receptor. Lower panel: Expression of *BmIRL* mRNA during follicle development. Expression of *Esp* and early chorion mRNA indicates the developmental period of vitellogenesis and early choriogenesis, respectively, and actin hybridizations are shown as control for the integrity of the RNA samples. Molecular weight sizes are 12 kb (BmIRL), 2 kb (ESP), 0.6 and 0.4 kb (early chorion), and 1.3 kb (actin). (Lower panel: Reprinted with permission from Swevers, L., Iatrou, K., **2003**. The ecdysone regulatory cascade and ovarian development in lepidopteran insects: insights from the silkmoth paradigm. *Insect Biochem. Mol. Biol. 33*, 1285–1297; © Elsevier.)

and Iatrou, 2003). The mRNA expression data, however, did not support the prediction for a role for BmIRL in the establishment of the autonomous portion of the program of differentiation at mid-vitellogenesis, as no differential expression was observed with respect to the stages, at which the autonomous capacity is induced in the follicular epithelium (Swevers and Iatrou, 2003). Because the *BmIRL* gene is mainly expressed in previtellogenic and early vitellogenic follicles (**Figures 13** and **14**), signaling through BmIRL is predicted to contribute to the early growth of ovarian follicles. The caveat for this conclusion, however, is that work for the detection of the receptor protein itself through Western analysis or immunolocalization, has not been carried out. Subsequent binding studies, to demonstrate that BmIRL is indeed the receptor for bombyxins, have also been inconclusive (E. Bullesbach, L.S., and K.I., unpublished data). Thus, the question of whether bombyxins are the sought-after inducing signal that establishes autonomy in follicular cells, remains unanswered.

1.3.3.3.2.1.2. Requirement for a decline in 20E signaling In *B. mori*, the transition from vitellogenesis to choriogenesis occurs during those stages of pharate adult development, when the hemolymph titers of ecdysteroids have already declined to low, nearly undetectable levels (Tsuchida *et al.*, 1987). Thus, it can be anticipated that the transition from vitellogenesis to choriogenesis is triggered by a decline in the primary 20E signaling in the developing vitellogenic follicles.

Experiments using the nonsteroidal ecdysone agonist tebufenozide have indeed shown that continuous signaling of the 20E pathway blocks ovarian development at mid to late vitellogenesis (Swevers and Iatrou, 1999). Because tebufenozide activates

the 20E signaling pathway at lower concentrations than 20E and persists in tissues much longer than the naturally occurring hormone, it causes prolonged 20E signaling that results in developmental arrest (Retnakaran *et al*., 1995).

When tebufenozide was injected in *Bombyx* developmentally arrested abdomens, ovarian development was induced and the follicles entered the developmental program of vitellogenesis with similar kinetics as in 20E-injected abdomens (Swevers and Iatrou, 1999). However, the transition from vitellogenesis to choriogenesis, monitored through the activation of chorion protein-encoding genes, an event associated with a decline in ecdysteroid titer, did not occur in tebufenozide-injected abdomens (see **Figure 4**). Thus, the experiments with tebufenozide have demonstrated unequivocally that vitellogenic follicles require a decline in 20E-signaling in order to progress towards choriogenesis.

1.3.3.3.2.1.3. Expression patterns of regulatory factors Comparison of the patterns of expression of regulatory factors between normally developing follicles obtained from intact animals and those of arrested follicles obtained from tebufenozide-injected abdomens has led to the identification of a regulatory cascade that is induced in follicular epithelium cells by the declining 20E titers and controls the transition from vitellogenesis to choriogenesis (Swevers and Iatrou, 1999).

At the top of the regulatory cascade stands the orphan nuclear receptor BmFTZ-F1 (Sun *et al*., 1994), which becomes expressed in the follicles at vitellogenic stages −18 to −7 (**Figure 13**). In *Drosophila*, βFTZ-F1 was shown to act as a competence factor during periods of low ecdysteroid signaling; between the periods of high ecdysteroid titers, tissues require low ecdysteroid titers and/or expression of FTZ-F1 in order to regain their capacity to respond properly to a consecutive pulse of 20E (Woodard *et al*., 1994; Broadus *et al*., 1999). Because BmFTZ-F1 seems to be the first factor induced by the declining ecdysone titers in *Bombyx* follicles, it may also function as a competence factor to control the transition from vitellogenesis to choriogenesis.

Interestingly, the induction of BmFTZ-F1 during vitellogenesis coincides with the highest expression levels of BmHR3A as well as with a drop in the expression levels of BmE75A (**Figure 13**; Swevers and Iatrou, 1999; Eystathioy *et al*., 2001; Swevers *et al*., 2002a). Moreover, in transient expression experiments employing *Bombyx*-derived tissue culture cells, BmHR3A and BmE75A function as activators and repressors, respectively, of reporter genes that harbor retinoic acid receptor-related orphan receptor elements (ROREs; binding elements for the HR3 and E75 orphan nuclear receptors) in their upstream regions (**Figure 15**; Swevers *et al*., 2002b). Because of the antagonistic activities displayed by BmHR3A and BmE75A, it was proposed that activation of RORE-dependent target genes depends on the relative expression levels of BmHR3A and BmE75A. It could indeed be demonstrated that during *Drosophila* metamorphosis, prolonged expression of E75 can prevent the activation of the *βFTZ-F1* gene by HR3 (White *et al*., 1997; Kageyama *et al*., 1997; Lam *et al*., 1997). The action of *Drosophila* HR3 and E75 is mediated through their interaction with RORE sites in the *βFTZ-F1* promoter. Whether similar sites also exist in the *Bombyx BmFTZ-F1* promoter is unknown at present, but it is tempting to speculate that the relative levels of BmHR3A and BmE75A determine the induction of BmFTZ-F1 during middle vitellogenesis in *Bombyx* follicles, according to a similar mechanism as that observed during metamorphosis in *Drosophila*.

The induction of BmFTZ-F1 is followed by changes in the expression of several other regulatory factors (**Figure 13**). Thus, at stages −12/−7, induction of the genes encoding the orphan nuclear receptor BmE75C and the adaptor protein BmSH3 occurs, while a concomitant strong decline is observed in the expression of the BmHR3 receptors (all isoforms). Furthermore, a strong decline in the levels of BmFTZ-F1 mRNA is observed at stages −6/−1, while the gene encoding the transcriptional regulator BmGATAβ becomes strongly upregulated at the same time (Drevet *et al*., 1995; Swevers and Iatrou, 1999; Eystathioy *et al*., 2001; Swevers *et al*., 2002a).

For most of the factors described above, however, their exact function in the program of follicular cell differentiation is unknown at present. *BmE75C* is initially induced in ovaries as an early response gene at the beginning of pupation but its expression is not sustained, and the transcription of this mRNA is rapidly downregulated (Swevers *et al*., 2002a). The subsequent (ecdysteroid-independent) increase in the expression of *BmE75C* mRNA (and protein) at stages −12 to +12 is consistent with a function as a regulator of the transition from vitellogenesis to choriogenesis. Because BmE75C acts as a transcriptional repressor in tissue culture cells (**Figure 15**; Swevers *et al*., 2002b), its function in follicular cells may be related to the shut-down of the vitellogenic program rather than the onset of choriogenesis.

BmSH3, a member of a family of conserved adaptor proteins characterized by three SH3 domains at the C-terminus and a lipid raft-targeting "SoHo" domain located at the N-terminus, was isolated

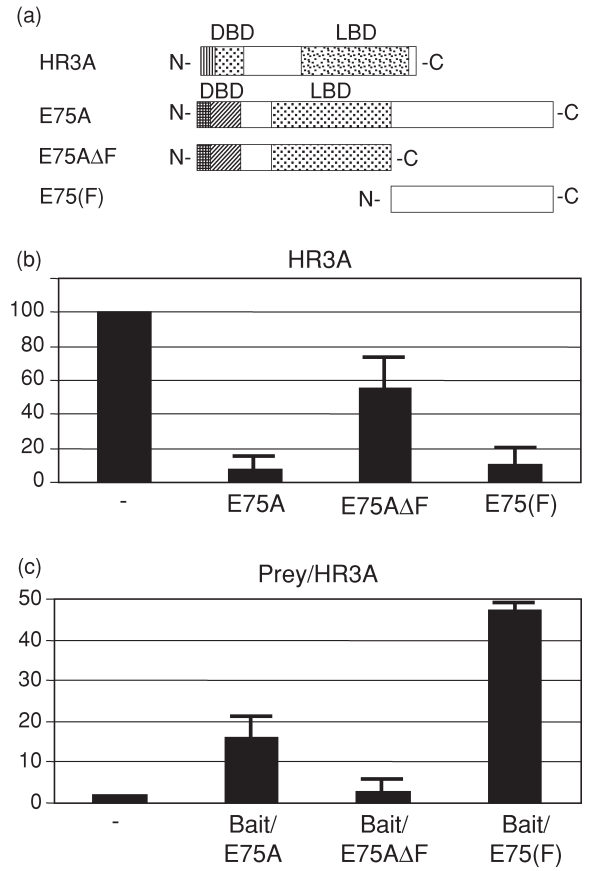


Figure 15 Functional interaction between the nuclear receptors BmHR3A and BmE75A. Panel (a): Schematic drawing of the nuclear receptors BmHR3A, BmE75A, and deletion mutants of BmE75A. In BmE75AΔF the C-terminal F-domain is deleted from the full-length receptor while BmE75(F) only consists of the F-domain. The two conserved domains of nuclear receptors, the DNA-binding domain (DBD) and ligand-binding domain (LBD) are indicated. Panel (b): BmE75A represses RORE-dependent reporter gene activation by BmHR3A in a silkmoth-derived cell line. Cells were transfected with RORE-dependent reporter plasmid and expression plasmids of *BmHR3A, BmE75A* and deletion mutants of *BmE75A*. ''−'': Transactivation by BmHR3A only; ''BmE75A'': Transactivation in the presence of BmHR3A and BmE75A; ''BmE75AΔF'': Transactivation in the presence of BmHR3A and BmE75AΔF; ''BmE75(F)'': Transactivation in the presence of BmHR3A and BmE75(F). Values of reporter gene activity are expressed as % of the activity obtained in the presence of BmHR3A only (''−'' column). Note that the F-domain of BmE75 is capable to repress transactivation by BmHR3A, due to interactions with the C-terminal activation function of BmHR3A (Swevers *et al.*, 2002b). Panel (c): Interactions between BmE75A and BmHR3A in yeast two-hybrid assays. Fusions between *BmE75A* or its deletion mutants and the DNA-binding domain of *Gal4* (''Bait'' proteins) were tested for interaction with a fusion between *BmHR3A* and the activation domain of *Gal4* (''Prey'' protein). In ''−,'' the empty ''bait'' vector was tested for interaction with Prey/HR3A. Relative reporter gene activities are expressed as percentages of the activity observed for the control interaction between p53 and SV40 large T-antigen. Note that the interaction between BmE75A and BmHR3A occurs via the F-domain of BmE75A; other experiments have shown that the F-domain forms a complex with the C-terminal activation domain of BmHR3A (data not shown; Swevers *et al.*, 2002b).

in a yeast two-hybrid screen using as bait the proline-rich N-terminus of BmE75C (K. Ito, L. Swevers, and K. Iatrou, unpublished data). Mammalian homologs of BmSH3 are involved in the transduction of tyrosine kinase signaling and the regulation of actin cytoskeleton organization (Baumann *et al.*, 2000; Kioka *et al.*, 2002). Thus, based on its structural features, BmSH3 may be involved in the regulation of the changes in the actin cytoskeleton in the cells of the follicular epithelium at the transition from vitellogenesis to choriogenesis (e.g., loss of patency, assembly of the chorion secretory machinery).

Finally, the BmGATAβ transcription factor has been hypothesized to be a major regulator of choriogenesis (Skeiky and Iatrou, 1991; Skeiky *et al.*, 1994; Drevet *et al.*, 1994, 1995) (see Section 1.3.4). Regarding a possible mechanism for the regulation of *BmGATAβ* gene expression, it should be noted that RORE binding sites exist in its promoter, which are bound by the BmHR3A orphan nuclear receptor in gel retardation assays. Because BmGATAβ and BmHR3A show reciprocal expression patterns during follicle development, it has been suggested that BmHR3A could act as a repressor of BmGATAβ expression (Eystathioy *et al.*, 2001). Although BmHR3A is known to act as a constitutive activator, as was mentioned earlier, its activation function can be repressed by the co-expression of BmE75A (Swevers *et al.*, 2002b). Thus, a possible mechanism for the silencing of the *BmGATAβ* gene during vitellogenesis may involve the recruitment of BmHR3A/BmE75A complexes to the RORE sites in the *BmGATAβ* promoter.

1.3.3.3.3. Other insects In insects, in which vitellogenin uptake by ovarian follicles is regulated by JH, termination of vitellogenesis can, in principle, be accomplished by two mechanisms: first, by a decrease in JH production by the CA or, second, by an increase in JH degradation in the hemolymph.

In the cockroach, *Diploptera punctata*, termination of vitellogenesis can be associated with a decline in JH titer (Sutherland *et al.*, 1998). Ovarian follicles that are in different stages of oogenesis exert different effects on the activity of the CA. Thus, follicles in active vitellogenesis stimulate JH production, while those near maturity inhibit it (Sutherland *et al.*, 2000). During the progression of vitellogenesis, the target for the ovarian inhibitory hormonal factor in the cells of the CA is proposed to be the CYP4C7 cytochrome P450 enzyme, which metabolizes JH and JH precursors by β-hydroxylation (Sutherland *et al.*, 1998, 2000). Thus, the decline in JH titer that is necessary to initiate post-vitellogenic events in

Diploptera, is mediated through a feedback signal from the ovary to the CA, which represses JH synthesis. By contrast, mechanisms that involve JH degradation by esterases in the hemolymph are thought to play a more minor role in the termination of vitellogenesis in this species.

In other cockroaches such as *L. maderae*, control of vitellogenesis is thought to be dependent on the fluctuation of the JH titer through the interplay among JH-binding proteins and JH degrading enzymes (for a relevant discussion, see Engelmann and Mala, 2000).

1.3.3.4. Ecdysteroidogenesis and Resumption of Meiosis

During the progression of vitellogenesis, ecdysteroid synthesis occurs in the cells of the follicular epithelium (Lagueux *et al*., 1977). However, in lepidopteran and orthopteran insects, most of the ecdysteroids produced by the follicular epithelium are inactive conjugates of ecdysone precursors and metabolites, and only very small amounts of active 20E are detected (Mizuno *et al*., 1981; Tawfik *et al*., 1999). In these insects, the ecdysteroids are not secreted to the hemolymph but are transported to the developing oocyte, where they are presumably stored as "maternal ecdysteroids" for use during embryonic development (Lagueux *et al*., 1981; Mizuno *et al*., 1981).

In the orthopteran *L. migratoria*, *in vitro* experiments have also shown that ecdysone promotes the reinitiation of meiosis in the oocyte at the end of vitellogenesis (Lanot *et al*., 1988, 1990). During oocyte growth, the oocyte nucleus (germinal vesicle) is arrested at the prophase of the first meiotic division. At middle to late vitellogenesis, however, germinal vesicle breakdown (GVBD) is initiated by the action of ecdysone produced by the follicular cells. Ecdysone is proposed to act at the level of the oocyte membrane, where it stimulates the production of cAMP (Lanot *et al*., 1988, 1990). After GVBD, the mature egg arrests again and remains arrested, until fertilization or some other signal triggers meiotic completion and further development.

In *B. mori*, ovarian ecdysteroid synthesis is initiated during day 4 of adult development (Ohnishi and Chatani, 1977), corresponding to the mid to late vitellogenic stages −20 to −25. Experiments using the ecdysteroid synthesis inhibitor KK-42 have shown that decreased ovarian ecdysteroid production does not affect ovarian growth, while it has profound effects on fertilization, embryogenesis, and larval hatching (Kadono-Okuda *et al*., 1994). These results suggest that, as is the case with *Locusta*, ovarian ecdysteroids are involved in the promotion of meiotic maturation in *Bombyx*. By contrast, since the experiments using the ecdysone agonist tebufenozide have shown that termination of vitellogenesis and initiation of choriogenesis requires a decline in 20E signaling (Swevers and Iatrou, 2003; Section 1.3.3.3.2.1.2), it is clear that the ecdysteroids produced by the follicular epithelium are not involved in the (autocrine or paracrine) regulation of follicle growth.

Interestingly, ecdysteroid synthesis in silkmoth follicles coincides with the expression of the nuclear receptor BmFTZ-F1, which also becomes expressed in follicular cells around stage −20 (**Figure 13**) (Swevers and Iatrou, 2003). BmFTZ-F1 is the insect homolog of the vertebrate nuclear receptor steroidogenic factor 1 (SF-1), which has essential roles in the differentiation of steroidogenic organs (gonads and adrenals) in vertebrates (Val *et al*., 2003). The SF-1 protein was originally isolated by its capacity to interact with and activate the promoters of steroidogenic enzymes (Rice *et al*., 1991; Morohashi *et al*., 1992). To deduce whether BmFTZ-F1 has a similar role in the regulation of ecdysteroidogenesis in ovarian tissue of *Bombyx*, the isolation of the genes encoding ecdysteroidogenic enzymes and the characterization of their promoter elements will be required.

In contrast to orthopteran and lepidopteran insects, the vitellogenic ovaries of dipteran insects (*Drosophila* and *Aedes*) synthesize significant amounts of 20E that are secreted into the circulation to stimulate vitellogenin synthesis in the fat body (Bownes, 1986; Raikhel *et al*., 1999; see also **Chapters 1.2**, **1.5**, and **3.9**). In *Drosophila*, the site of synthesis is the cells of the follicular epithelium, as demonstrated by the detection of the mRNA of the ecdysteroidogenic enzyme encoded by the *disembodied* (*dis*) gene through *in situ* hybridization (Warren *et al*., 2002) (see **Chapter 1.2**). In *Aedes* and *Phormia*, the synthesis of 20E is stimulated by neurohormones (see Sections 1.3.3.1.1.2 and 1.3.3.3.1.2), presumably through a cAMP-dependent mechanism (Brown *et al*., 1998; Manière *et al*., 2002). It has also been proposed that 20E stimulates ovarian growth by an autocrine/paracrine mechanism, although the evidence for this is not entirely convincing (Buszczak *et al*., 1999; see Section 1.3.3.1.1.1.3). However, whether ecdysteroids produced by the ovary of dipteran insects are also involved in the regulation of reinitiation of meiosis is not known. In *Drosophila*, as in *Locusta*, ovarian oocytes are arrested in prophase I during vitellogenic growth and undergo GVBD upon maturation (Page and Orr-Weaver, 1997; Chen *et al*., 2000) (see **Chapter 1.2**). The mature oocytes become arrested in metaphase I, but are released from the arrest when the eggs pass through the oviduct into the uterus (Heifetz *et al*., 2001) (see also Section

1.3.5). Thus, it can be hypothesized that the ecdysteroids produced by the follicular epithelium in *Drosophila* are also involved in the stimulation of the transition from prophase I to metaphase I during oocyte maturation.

1.3.3.5. Osmotic Swelling and Loss of Patency

The mechanism that triggers the loss of patency at the end of vitellogenesis has been studied mostly and is best understood in the lepidopteran *H. cecropia* (Telfer and Woodruff, 2002). In this species, loss of patency occurs through osmotic swelling (50% increase in cellular volume) of the cells of the follicular epithelium and concomitant closure of the intercellular spaces among the epithelial cells. This process is triggered by a rise in the cAMP content of the follicular cells (Wang and Telfer, 1996), which results in the activation of protein kinase A, and changes in the electrophysiological properties of the cells (increase of chloride ion conductance and potassium ion uptake, acidification of the cytoplasm, hyperpolarization) that result in fluid uptake (Wang and Telfer, 1998a). Interestingly, the rise in cAMP levels and loss of patency can also be effected by pertussis toxin, an inhibitor of the α subunit of inhibitory G proteins (Giα) (Wang and Telfer, 1998b). Thus, based on the assumption that Giα is a component of a conventional heterotrimeric G protein, it has been suggested that during vitellogenesis, cAMP levels in the epithelium remain low through signaling by an unknown GPCR that couples with Giα-containing G protein complexes. At the end of vitellogenesis, however, a decrease in signaling (loss of ligand or decrease in ligand sensitivity) through the hypothetical GPCR is postulated to lead to increased levels of cAMP, which result in osmotic swelling and loss of patency in the follicular epithelium (Wang and Telfer, 1998b). The transformation of the follicular epithelium, however, is thought not to be triggered by extrafollicular factors circulating in the hemolymph but by developmental changes occurring spontaneously in each follicle as it reaches a length of 2 mm (Wang and Telfer, 1996, 1998b).

The activation of protein kinase A in homogenates of follicles also results in the phosphorylation of a 32 kDa protein that has been speculated to represent ribosomal protein S6 (Wang and Telfer, 1996, 2000). However, cell-permeable cAMP analogs that inhibit phosphorylation of the 32 kDa protein, were found to be capable of triggering termination of vitellogenesis in intact follicles, suggesting that the phosphorylation of this protein is not involved in the pathway that terminates vitellogenesis.

Because the cells of the follicular epithelium and the oocyte communicate through gap junctions that allow exchange of ions and small solutes (Telfer and Woodruff, 2002), osmotic swelling occurs both in the cells of the follicular epithelium and the oocyte. However, the process seems to be triggered in the follicular cells because of their high levels of protein kinase A. Osmotic swelling occurs immediately (within 30 min after the rise in cAMP) in the follicular cells, while a significant delay exists for the oocyte. The delay in the swelling of the oocyte probably contributes to the requirement for a mechanical loosening of the vitelline envelope (see Section 1.3.3.8 below), which would otherwise prevent the immediate expansion of the oocyte volume (Wang and Telfer, 1998a).

1.3.3.6. Patterning and Movements of the Follicular Epithelium

The patterning and morphogenetic movements of the follicular epithelium at the transition from vitellogenesis to choriogenesis in *D. melanogaster* have been subject to several excellent recent reviews (Ray and Schüpbach, 1996; Van Eeden and St. Johnston, 1999; Dobens and Raftery, 2000; Riechmann and Ephrussi, 2001; Rørth, 2002) (see **Chapter 1.2**). Only a brief outline will be presented here. However, very little to no information on these important processes exists for other insects.

At the beginning of *Drosophila* vitellogenesis (stage 9), the follicular epithelium is organized in three domains: (1) a small cluster of border cells that has delaminated from the anterior pole of the follicle and migrates between the cells of the nurse cell complex towards the oocyte surface, (2) a squamous epithelium that covers the nurse cell cluster, and (3) a columnar epithelium that covers the oocyte. At the end of vitellogenesis (stage 10B), the nurse cells start dumping their contents into the oocyte (see below), while the most anterior cells of the columnar epithelium move centripetally between the nurse cell cluster and the oocyte to seal the anterior surface of the oocyte. The centripetally migrating cells stop their migration when they meet the border cells. The leading cells of the centripetally moving population of follicular epithelium cells, together with the border cells, form the micropyle of the eggshell, while the remainder of the centripetally moving cells build the operculum. During stage 11, the main body follicle cells also stretch along the anterior–posterior axis to accommodate the increase in oocyte volume during nurse cell dumping (Spradling, 1993; Dobens and Raftery, 2000) (see **Chapter 1.2**).

The patterning and movements of the follicular epithelium are controlled by several autocrine and paracrine signaling pathways. Thus, the "Janus Kinase/Signal Transducer and Activator of Transcription" (JAK/STAT) pathway is responsible for border cell specification and migration between the cells of the nurse cell complex (Castelli-Gair Hombría and Brown, 2002). Furthermore, migration toward the oocyte is guided by spatial cues that are detected by the *Drosophila* homologs of the mammalian platelet-derived growth factor receptor/vascular endothelial growth factor receptor (PDGFR/VEGFR) and the epidermal growth factor receptor (EGFR) pathways (Rørth, 2002). The best known transcription factor necessary for border cell migration is Slow border cells (SLBO), a basic region/leucine zipper transcriptional activator related to the mammalian CCAAT/enhancer-binding protein (C/EBP) transcription factors (Montell, 2001). Interestingly, temporal cues for border cell migration seem to be initiated by components of the ecdysone receptor signaling, such as the USP nuclear receptor and the p160 class of the nuclear receptor coactivator Taiman (Tai; Montell, 2001). Nevertheless, the findings that *tai* or *usp* mutants show deficiencies in border cell migration do not necessarily prove an involvement of ecdysone, since both USP and Tai could act as components of other signaling pathways (see discussion in Section 1.3.3.1.1.1.3; also Swevers and Iatrou, 2003). From a comparative viewpoint, it should be noted that the specification of a group of apical follicular cells that migrate through the cells of the nurse cell complex to reach the oocyte surface (border cells), is specific to the order of the Diptera and does not occur in species of other insect orders (Büning, 1994).

The formation of anterior structures in the eggshell, mediated by the centripetally migrating and border cells, is controlled by the transforming growth factor β/Decapentaplegic (TGFβ/Dpp) and the epidermal growth factor (EGF) pathways (Twombly *et al*., 1996) (see **Chapter 1.2**). The cells at the leading edge of the centripetally migrating population produce Dpp during migration and micropyle formation (Twombly *et al*., 1996; Dobens *et al*., 2000). EGF signals, alternatively, emanate initially from the oocyte nucleus at the dorsal-anterior part of the oocyte, and are subsequently modulated by autocrine signaling in the dorsal-anterior follicular epithelium (Stevens, 1998; Van Buskirk and Schüpbach, 1999). Overexpression experiments have shown that correct patterning of the anterior part of the follicular epithelium/eggshell is dependent on the integration of both Dpp and EGF signaling (Dobens and Raftery, 2000).

1.3.3.7. Nurse Cell Dumping and Apoptosis

The process of rapid transport of the contents of the nurse cells to the oocyte at the end of vitellogenesis that is accompanied by programmed cell death in the nurse cells, has been described in detail and subjected to genetic analysis in *D. melanogaster* (review: **Chapter 1.2**). In other insect species, it is not known whether these processes are regulated by mechanisms analogous to those occurring in *Drosophila* or by different pathways.

In *Drosophila*, the nurse cell cytoplasm is rapidly transported to the oocyte during stages 10B and 11 of oogenesis. This results in the doubling of the oocyte volume in about 30 min. Concomitantly, circular streaming is initiated in the cytoplasm of the oocyte that effects the rapid mixing of the nurse cell contents with the cytoplasm of the oocyte (Mahajan-Niklos and Cooley, 1994).

The transport of the contents of the nurse cell cytoplasm is regulated by the actin cytoskeleton, which performs two functions. First, the cortical actin cytoskeleton together with a myosin motor generates the driving force required to expel the nurse cell cytoplasm (Wheatley *et al*., 1995; Edwards and Kiehart, 1996). Second, actin bundles anchor the nurse cell nuclei to the membrane, thus preventing them from blocking the path of cytoplasmic flow through the ring canals (Cooley *et al*., 1992; Cant *et al*., 1994; Matova *et al*., 1999).

The process of dumping of the cytoplasmic contents of the nurse cells to the oocyte is closely associated with the initiation of programmed cell death (apoptosis) in the nurse cell complex. Loss of function of the *dcp-1* gene, which encodes an effector caspase involved in apoptosis, results in defects in nurse cell dumping, suggesting that apoptosis is a necessary event for the final transport of the nurse cell contents into the oocyte (McCall and Steller, 1998). After the majority of the cytoplasm is lost from the nurse cells, DNA breakdown occurs in the nurse cell nuclei during stage 13, and the nurse cell remnants are removed via phagocytosis by the follicular cells at stage 14 (Foley and Cooley, 1998; Cavaliere *et al*., 1998; Nezis *et al*., 2000). The activities of the regulators of the death process (interplay between caspases and caspase inhibitors) are proposed to be temporally and spatially controlled, since the oocyte must be protected from the death process that occurs in the connected nurse cells (Peterson *et al*., 2003).

Nurse cell dumping and apoptosis must also be tightly coordinated with the morphogenetic movements in the follicular epithelium, to allow the appropriate formation of a continuous epithelium that

will seal the oocyte completely for deposition of the chorion or eggshell. The signaling pathways, between the germline and the cells of the follicular epithelium that coordinate these processes, include the c-Jun-NH_2-terminal kinase (JNK) pathway (Dequier *et al*., 2001; Dobens *et al*., 2001) and others that may involve GPCRs and cAMP signaling (Schneider and Spradling, 1997; Lannutti and Schneider, 2001; Pathirana *et al*., 2001).

1.3.3.8. Vitelline Membrane Synthesis

1.3.3.8.1. Dipteran insects

***1.3.3.8.1.1.* Drosophila melanogaster** To date, four *Drosophila* vitelline membrane protein (VMP) genes have been cloned, *VM26A.1, VM26A.2, VM34C*, and *VM32E* (Higgins *et al*., 1984; Mindrinos *et al*., 1985; Burke *et al*., 1987; Popodi *et al*., 1988; Gigliotti *et al*., 1989). While *VM26A.1, VM34C*, and *VM26A.2* are expressed continuously from stages 8 to 10, *VM32E* is considered to be a "late" gene and is expressed only at stage 10. Furthermore, *VM34C* and *VM32E* are not expressed in the polar follicular cells, in contrast to *VM26A.1* and *VM26A.2*. However, upon synthesis and secretion, both proteins have been postulated to diffuse towards the poles of the follicles (Andrenacci *et al*., 2001).

For the *VM26A.1* and *VM32E* genes, analysis of their promoter regions has been carried out in transgenic flies, and *cis*-regulatory elements for temporal and spatial regulation as well as expression levels have been delineated (Gargiulo *et al*., 1991; Jin and Petri, 1993; Cavaliere *et al*., 1997; Andrenacci *et al*., 2000). However, no *trans*-acting factors that regulate the expression of the *VMP* genes have been identified thus far.

Immuno-electron microscopy and confocal immunofluorescence studies have shown that yolk proteins (Yps) and VMPs are co-secreted by the cells of the follicular epithelium (stages 9 and 10 of oogenesis). While the VMPs are incorporated in the incomplete vitelline membrane (VM) matrix, the YPs diffuse through gaps in the vitelline layer to reach the oocyte surface. During stage 10B, the incomplete parts of the VM fuse to form a continuous layer that covers completely the oocyte surface (Margaritis, 1985; Trougakos *et al*., 2001).

All VMPs become localized in the VM during choriogenesis. However, during the later stages, VM32E (but not the other VMPs) is capable of moving from the VM layer to the endochorion (which is secreted during stages 11–14), indicating its participation in the formation of endochorion structures (Andrenacci *et al*., 2001). The VMPs also undergo proteolytic processing during choriogenesis (Pascucci *et al*., 1996), but the functional relevance of this finding remains obscure.

The VMPs are characterized by a conserved hydrophobic domain of 38 aa, the VM domain (Scherer *et al*., 1988) which is necessary for their assembly in the VM. Alternatively, the domain responsible for the localization of the VM32E protein to the endochorion during choriogenesis resides at the C-terminus of the protein (Andrenacci *et al*., 2001).

In the female sterile mutation *fs(2)QJ42*, the follicles fail to accumulate VM26A.2, and this results in the formation of an altered VM, onto which the endochorion layer collapses during stage 14 (Savant and Waring, 1989). Thus, the preformation of a properly assembled VM is a prerequisite to the formation of a stable chorion structure (Pascucci *et al*., 1996).

Another genetic locus implicated in vitelline membrane and chorion formation in *Drosophila* is the *defective chorion-1* (*dec-1*) locus (Waring *et al*., 1990). The *dec-1* gene encodes multiple protein products that are generated by alternative splicing and protein processing. Three proproteins, fc177, fc125, and fc106, are produced that share a common N-terminus but are distinguished by differential C-termini. Following their secretion, the proproteins undergo distinct extracellular maturation pathways: fc106 is processed during stage 10B into s80, which undergoes an additional cleavage that generates s60 during stage 14; fc125 is initially cleaved to a 125 kDa protein at stage 10A and subsequently to 110 and 95 kDa proteins during stage 10B; and fc177 undergoes a cleavage to a 125 kDa and a second one that yields an 85 kDa protein during stages 12 and 13, respectively (Noguerón and Waring, 1995; Noguerón *et al*., 2000). The DEC-1 proteins are synthesized concomitantly with the VMPs and accumulate extracellularly between the follicular epithelium and the oocyte. The VM functions both as the site where the proteolytic cleavage products are generated and as a reservoir for the release of the different cleavage products to the oocyte and distinct regions of the chorion, where they presumably exert unique functions (Noguerón *et al*., 2000). Mutants of *dec-1* fail to organize the endochorion, which, as is the case of the *fs(2)QJ42* mutation, collapses into the VM during late choriogenesis (Waring *et al*., 1990).

Recently, the distinct functions of the three DEC-1 proproteins were also dissected genetically. Using specific *dec-1* mutations, in conjunction with introduced *dec-1* transgenes, it was shown that gross morphological abnormalities occur only in the absence of fc177, although all three proproteins are essential for female fertility. It was proposed that fc177 acts as a scaffolding protein necessary for the

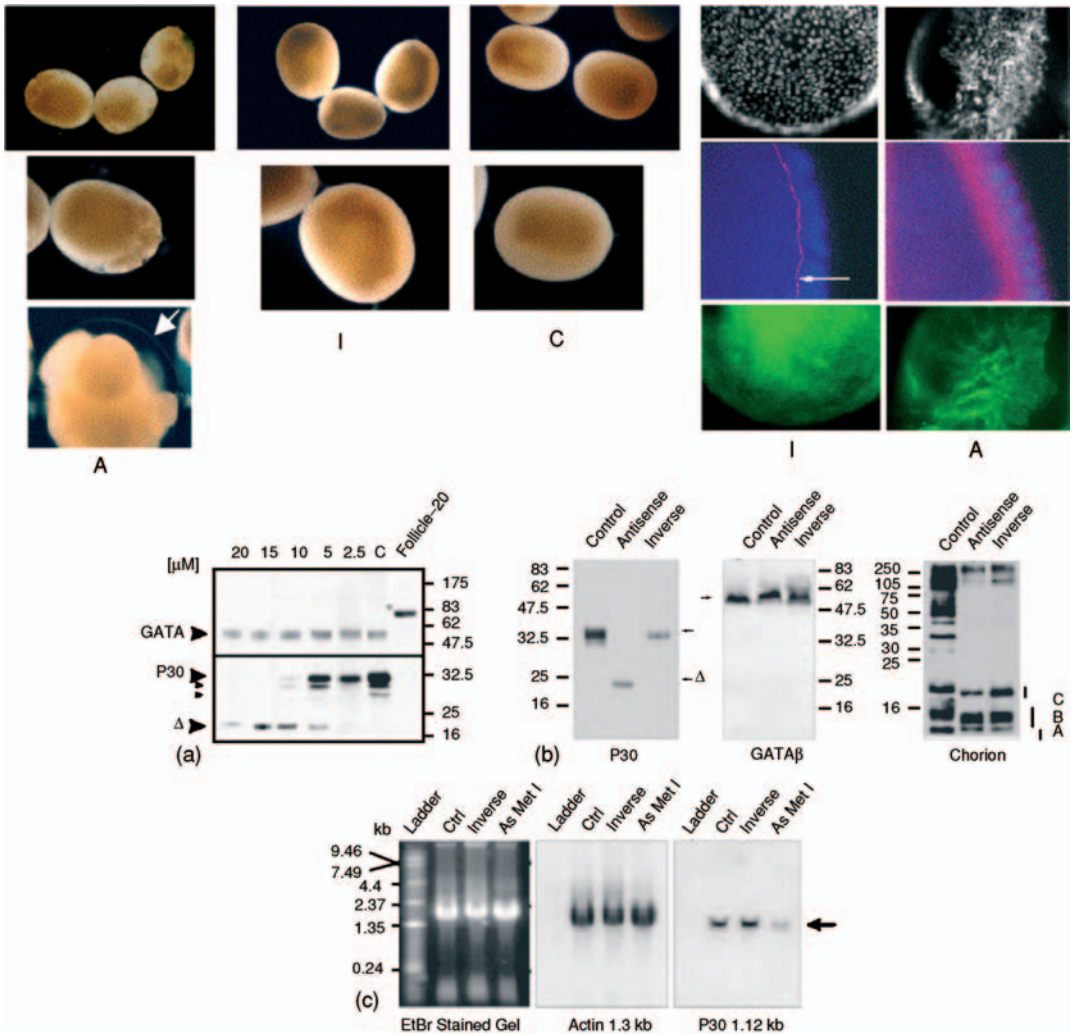


Figure 16 Functional analysis of the *BmVMP30* gene of *Bombyx mori* that encodes a putative member of the insect vitelline membrane protein family. Follicles were treated with antisense or control (inverse) oligonucleotides and the effects of the treatments on the morphology of the follicles and the expression of *BmVMP30* and other genes were assessed. Upper left panel: Light microscopy observations of the integrity of the follicular epithelium and the oocyte of developing follicles following treatment with 15 μM of antisense oligonucleotide (panel A) or control (inverse) oligonucleotide (panel I) or without treatment (panel C) for 48 h *in vitro*. Note the abnormal distribution of the yolk within the antisense oligonucleotide-treated follicles (white arrow in bottom panel of panel A). Upper right panel: Fluorescence microscopy observations of the integrity of the follicular epithelium and the oocyte of developing follicles following treatment with 15 μM of antisense oligonucleotide (panel A) or inverse oligonucleotide (panel I) for 48 h *in vitro*. Follicles were stained with DAPI (top) or stained with fluorescent anti-BmVMP30 (middle) or anti-tubulin (bottom) antibody complexes. While follicles treated with inverse oligonucleotide retain their wild-type appearance, disruptions of the follicular epithelium occur after treatment with antisense oligonucleotide. In panel A, note the disruption of the follicular epithelium characterized by gaps in the nuclear staining (top right), diffuse localization of BmVMP30 epitopes within the follicular cells (middle right), and a nonhomogeneous tubulin staining (bottom right). The normal location of BmVMP30 and the vitelline membrane (wild-type appearance) is marked by a white arrow in the left middle panel (bar = 50 μm). Bottom panel: Western and Northern blot analysis of the expression of *BmVMP30* and other genes after treatment of follicles with BmVMP30 antisense and control oligonucleotides. (a) Western blot demonstrating the effects of different concentrations (2.5 to 20 μM) of BmVMP30 antisense oligonucleotide on BmVMP30 (P30) protein expression after *in vitro* culture of middle/late (stage ''−20'') vitellogenic follicles for 48 h. No BmVMP30 was initially detected in mid-vitellogenic follicle ''−20'' prior to culture (right-most lane). Arrows point to truncated BmVMP30 proteins presumed to originate from the use of alternative translation start sites; ''Δ'' indicates the presence of the putative truncated ΔBmVMP30 protein. While the antisense oligonucleotides had profound effects on BmVMP30 protein expression (lower), no changes were observed in the expression of the 55 kDa BmGATAβ protein (upper). Note that the BmGATAβ antiserum recognizes an additional unknown ~70 kDa protein in ''−20'' follicles (star). C, control ''−20'' follicles cultured *in vitro* for 48 h in the absence of antisense oligonucleotide. Molecular weight markers are indicated at right. (b) Western blot demonstrating the effects of 15 μM of antisense or inverse oligonucleotide on BmVMP30 (P30) (left), BmGATAβ (middle), or chorion protein (right) expression after *in vitro* culture of middle/late vitellogenic follicles (stage ''−20'') for 48 h. The presence of BmVMP30, ΔBmVMP30 (Δ), and BmGATAβ proteins are indicated by arrowheads while the different chorion protein families (A, B, C) are indicated by bars. Numbers refer to molecular weights (kDa) of protein markers. (c) Northern blot analysis demonstrating the effects of 15 μM of

erection of the chorion structure (Mauzy-Melitz and Waring, 2003).

The VM represents also a storing site for secreted follicular epithelium cell products involved in embryonic pattern formation (see **Chapter 1.2**). Extracellular proteins that signal the patterning of the embryo are anchored in the VM and participate actively in the VM assembly. The Nudel protease, for example, a large modular protein with a trypsin-like serine protease domain, functions both in the generation of the extracellular signal that determines the dorsoventral axis of the embryo, and the cross-linking of the vitelline membrane (LeMosy *et al*., 1999; LeMosy and Hashimoto, 2000). Similarly, the *fs(1)Nasrat* and *fs(1)polehole* genes are not only required for the generation of the ligand for the local activation of the Torso (TOR) receptor at the termini of embryos, but also have structural roles in the biogenesis of the VM (Cernilogar *et al*., 2001).

1.3.3.8.1.2. **Aedes aegypti** Three genes encoding VMPs have been cloned in *A. aegypti*, *15a-1*, *15a-2*, and *15a-3* (Lin *et al*., 1993; Edwards *et al*., 1998). All are characterized by a short 46 aa sequence that bears similarities with the VM domain of the *Drosophila* VMPs. The expression patterns of the vitelline membrane proteins during oogenesis overlap those of the YPs. *In situ* hybridization has also shown that the different *VMP* genes are expressed in spatially distinct domains of the follicular epithelium: *15a-1* and *15a-3* are expressed in the middle and the posterior regions of the follicle, while *15a-2* is expressed over the entire surface (Edwards *et al*., 1998). Finally, a moderate stimulation of expression levels of the VMPs could be achieved by addition of high concentrations 20E (Lin *et al*., 1993; Edwards *et al*., 1998).

1.3.3.8.2. Lepidopteran insects In general, and in contrast to chorion formation (Section 1.3.4), very little is known about vitelline membrane formation in lepidopteran insects at the molecular level. In the best studied lepidopteran model, the domesticated silkmoth *B. mori*, for example, only one vitelline membrane protein gene has been isolated and characterized in detail (see below).

1.3.3.8.2.1. **Bombyx mori** A cDNA clone, *BmVMP30*, encoding a putative VMP has been isolated from a screen for genes that are differentially expressed with respect to stages −35/−1 (Kendirgi *et al*., 2002). BmVMP30 is a 30 kDa protein that is synthesized and secreted by the cells of the follicular epithelium during late vitellogenesis (stages −18 to −1). The protein accumulates between the oocyte surface and the follicular epithelium, where the VM is located (**Figure 16**), and remains associated with the egg envelope during choriogenesis and after egg-laying. However, after fertilization no BmVMP30 protein could be detected by Western blot analysis in the eggshell fraction of the developing embryos. Thus, the temporal and spatial expression pattern of BmVMP30 during and after oogenesis is consistent with the behavior expected for a VMP (Kendirgi *et al*., 2002). Although BmVMP30 does not have significant sequence identity with any *Drosophila* or mosquito VMPs, including the VM domain, it does share with them a distinct overall proline distribution, a similar general hydropathy profile and the lack of introns in the gene structure (Kendirgi *et al*., 2002).

Strong knockdowns of *BmVMP30* gene expression, achieved by antisense oligonucleotide technology, resulted in the appearance of a severe phenotype suggesting disruption of the integrity of the follicular epithelium, while no interference was observed with the initial steps of the program that controls the activation of chorion protein genes (**Figure 16**; Kendirgi *et al*., 2002). Because the knockdown experiments were carried out in *in vitro* culture, the long-term effects on follicle development and eggs (e.g., completion of choriogenesis) could not be assessed.

The expression of *BmVMP30* requires down-regulation of the 20E signaling, as its mRNA is not observed in ovaries that are treated with the 20E agonist tebufenozide (Swevers and Iatrou, 1999; see also Section 1.3.4.1.1.2). Interestingly, *BmVMP30* gene expression becomes upregulated during follicular development concomitantly with that of the orphan nuclear receptor BmFTZ-F1, suggesting a possible regulation of the expression of *BmVMP30* by BmFTZ-F1 (Swevers and Iatrou, 1999, 2003).

1.3.4. Choriogenesis

The deposition of the eggshell or chorion occurs when the cells of the follicular epithelium undergo their terminal differentiation and become devoted to the

antisense (AsMet1) or inverse oligonucleotide on BmVMP30 (P30) and actin mRNA expression after *in vitro* culture of middle/late vitellogenic follicles (stage ''−20'') for 48 h. The specific reduction of BmVMP30 mRNA by antisense oligonucleotide treatment is indicated by an arrow. RNA molecular weight markers are indicated on the left. EtBr, ethidium bromide; Ctrl, control. (Reprinted by permission of Federation of the European Biochemical Societies from An ovarian follicular epithelium protein of the silkworm (*Bombyx mori*) that associates with the vitellise membrane and contributes to the structural integrity of the follicle, by Kendirgi, F., Swevers, L., Iatrou, K., *FEBS Letters* 524, 59–68, © 2002.)

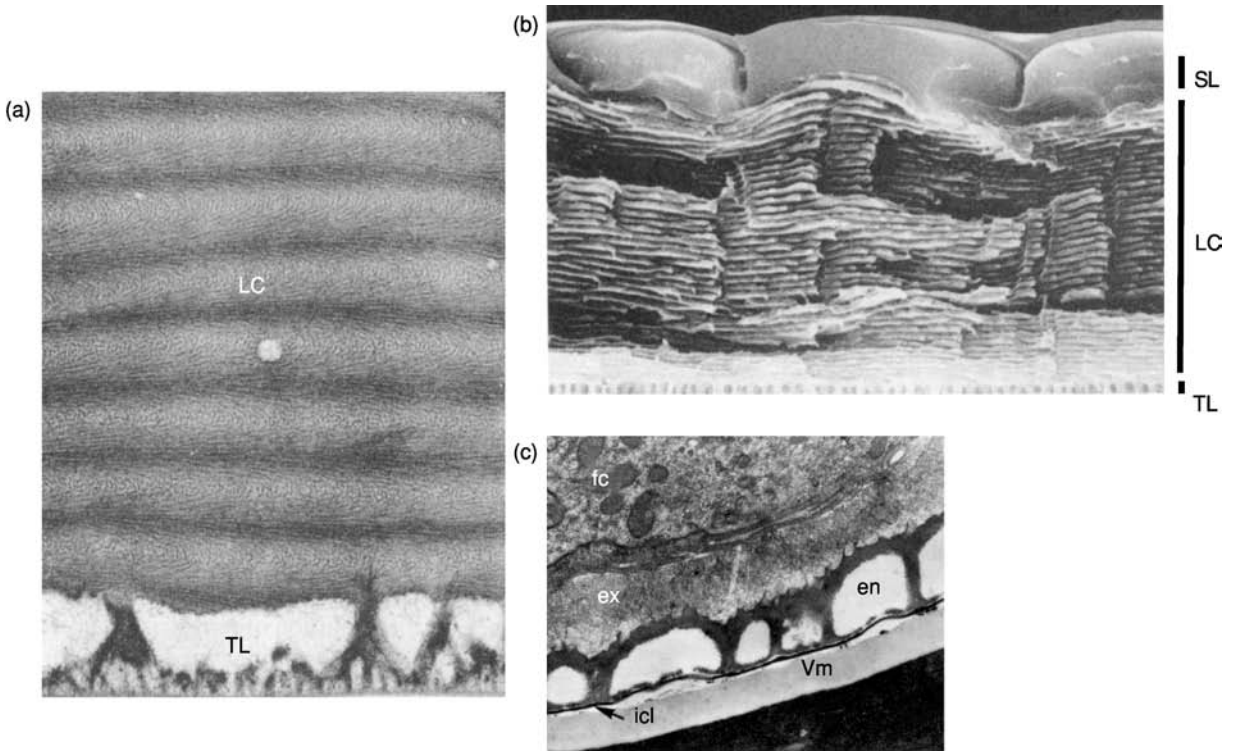


Figure 17 Chorion structure of eggs from *Hyalophora cecropia* (a), *Bombyx mori* (b), and *Drosophila melanogaster* (c). (a) Transmission electron micrograph of an immature chorion from the silkmoth *H. cecropia*. The trabecular layer (TL) is at the bottom with a portion (approximately 6% of total) of the helicoidal lamellae (lamellar chorion, LC) above. Magnification = 30 000×. (Reprinted from Regier, J.C., Friedlander, T., Leclerc, R.F., Mitter, C., Wiegmann, B.M., **1995**. Lepidopteran phylogeny and applications to comparative studies of development. In: Goldsmith, M.R., Wilkins, A.S. (Eds.), Molecular Model Systems in the Lepidoptera. Cambridge University Press, Cambridge, pp. 107–137, with permission from Cambridge University Press.) (b) Scanning electron micrograph of a transverse rip through the mature eggshell of *B. mori*. Indicated are the different chorion layers: the small trabecular layer (TL) is located at the side of the vitellin membrane and the oocyte, while the bulk of the chorion consists of multiple horizontal lamellae (LC). At the top (external surface), a few specialized surface lamellae (SL) are deposited. Magnification = 2000×. (Reprinted from Kafatos *et al.*, 1985, with permission from Cold Spring Harbor Laboratory Press.) (c) Transmission electron micrograph of a section of a stage 14 follicle of *D. melanogaster* showing the structure of the eggshell. The oocyte (o), vitellin membrane (Vm), inner chorionic layer (icl), endochorion (en), exochorion (ex), and overlying follicle cell (fc) are indicated. Magnification = 150 000×. (Reprinted from Spradling, A.C., **1993**. Developmental genetics of oogenesis. In: Bate, M., Martinez Arias, A. (Eds.), The Development of *Drosophila melanogaster* vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1–70, with permission from Cold Spring Harbor Laboratory Press. Photo courtesy of Drs. G. Waring and A. Mahowald.)

synthesis and secretion of the chorion proteins, which subsequently become assembled extracellularly into a large macroscopic structure (Orr-Weaver, 1991; Kafatos *et al.*, 1995). The insect eggshell is a complex structure that protects the developing embryo in a hostile environment (**Figure 17**). Although water loss should be minimized to prevent desiccation, the eggshell should provide sufficient gas exchanges to allow embryo respiration. Eggshells therefore contain specialized regions that facilitate gas exchange between the embryo and the environment. Examples of such specialized structures for air transport include the dorsal appendages at the dorsal-anterior part of the egg surface, and the aeropyles at the posterior pole of the *Drosophila* egg. The eggshell also contains other specialized structures: at the anterior end are located both the micropyle, that forms a channel through the eggshell that allows the sperm to enter the oocyte surface for fertilization, and the operculum, which functions as the door through which the larva will exit the eggshell (Spradling, 1993).

Choriogenesis has been studied mostly in dipteran and lepidopteran insects, most notably in *D. melanogaster* and *B. mori* (Kafatos *et al.*, 1995). The process of choriogenesis differs substantially between flies and moths: the structure of the *Drosophila* chorion is relatively simple and is composed of six major and 14 minor proteins (Kafatos *et al.*, 1985, 1995); the silkmoth chorion, alternatively, is a more complex structure, consisting of more than 100 different polypeptides and becoming assembled in a stepwise fashion over a period of approximately 2 days (Kafatos *et al.*, 1977, 1985, 1995). Similar complex chorion structures are

observed in other lepidopteran insects of the Bombycoidea superfamily (Regier *et al*., 1995). Recently, the structure of the chorion of a coleopteran insect, the Colorado potato beetle *Leptinotarsa decemlineata*, has been also presented (Papassideri *et al*., 2003).

The evolution of the structure of the chorion in lepidopteran insects has also been reviewed (Regier *et al*., 1995), and the principles that govern the molecular architecture of helicoidal proteinaceous eggshells of the Lepidoptera have described in detail by Hamodrakas (1992). Interestingly, Hamodrakas and his collaborators have provided evidence that the silkmoth eggshell may be a natural protective amyloid (Iconomidou *et al*., 2000), and described how these amyloids are formed *in vitro* via a liquid crystalline mesophase (Hamodrakas *et al*., 2004; see also **Chapter 4.2**).

After secretion of the chorion proteins and assembly of the eggshell, the latter becomes stabilized through an extensive crosslinking process that involves peroxidase activity. Peroxidase catalyzes the formation of di- and tri-tyrosyl crosslinks among chorion proteins, a process that strengthens the eggshell. The peroxidase activity is initiated through the production of hydrogen peroxide by the cells of the follicular epithelium (Margaritis, 1985; Han *et al*., 2000). In mosquitos, the process of chorion tanning (melanin formation) requires tyrosine as well as the action of the enzymes phenol oxidase and dopa decarboxylase (Li, 1994; Ferdig *et al*., 1996).

1.3.4.1. Chorion Genes and Regulation of Chorion Gene Expression

The early studies on the topic have been reviewed extensively in the past (Regier *et al*., 1995; Eickbush and Izzo, 1995; Goldsmith and Kafatos, 1984; Kafatos *et al*., 1987, 1995). In this section, most recent information related to the structure of chorion genes and the regulation of their expression is reviewed, which has been obtained through the study of the two major insect models, the fruit fly *D. melanogaster*, and the silkmoth, *B. mori*.

1.3.4.1.1. ***Drosophila melanogaster*** Starting from the VM and moving in an outward fashion, the *Drosophila* chorion consists of four layers, the wax layer, the innermost chorionic layer (ICL), the endochorion, and the exochorion (**Figure 17**; Spradling, 1993; Trougakos and Margaritis, 1998). The chorion consists mainly of six major and 14 minor proteins. The genes that encode two major and four minor proteins (s36, s37, s38, A, B, C) are clustered on the X chromosome (region 7F1; Parks *et al*., 1986; Orr-Weaver, 1991), while those for the four other major proteins (s15, s16, s18, s19) are encoded in a cluster on chromosome 3 (region 66D11-15; Kafatos *et al*., 1985, 1995). The general features of the chorion genes are their organization in tandem arrangement in both clusters (**Figure 18**), the presence of a small intron near the 5′-end of each gene, and the presence of repeated sequences in the protein coding regions.

The genes on the X cluster are considered "early" genes, since they are expressed during stages 11–13 of oogenesis, when the ICL and endochorion components are produced. The genes on the third chromosome cluster, however, are expressed during stages 13 and 14, when the exochorion is deposited and, thus, represent "late" genes (Mariani *et al*., 1988; Tolias and Kafatos, 1990). While the chorion proteins play a structural role in chorion assembly, it has also been proposed that s38 corresponds to the eggshell peroxidase enzyme (Keramiris *et al*., 1991). The organization of the chorion gene clusters is conserved in the Drosophilidae family and in the more distantly related medfly *Ceratitis capitata* (Vlachou and Komitopoulou, 2001).

Recently, a novel chorion protein of *Drosophila*, Femcoat, was isolated. The *femcoat* gene is expressed in the follicular cells during stages 12B to 14A of oogenesis. Double-stranded RNA-mediated RNA interference has shown that Femcoat is essential for the formation of the endochorion (Kim *et al*., 2002).

1.3.4.1.1.1. ***Chorion gene amplification*** To accommodate the high levels of gene expression that are required for the production of the chorion during a period of only 1–2 h (stages 11–14 of oogenesis), gene amplification occurs in both chorion gene clusters of *Drosophila* (Kafatos *et al*., 1985; Kalfayan *et al*., 1985). The X-chromosome and third chromosome clusters are amplified 15- to 20-fold and 60- to 100-fold, respectively, above the remainder of the genome of the already polyploid (see below) cells of the follicular epithelium (Calvi and Spradling, 1999).

The selective increase in gene copy number occurs through repeated initiation of replication forks at selected sites in these loci (**Figure 18**). Because replication forks continue to move outwards as choriogenesis progresses, a gradient of amplified DNA extending 40–50 kb to either side of the chorion gene cluster is generated that can be observed as "onion-like" structures in the electron microscope (**Figure 18**) (Orr-Weaver, 1991; Royzman and Orr-Weaver, 1998).

Genetic analysis in transgenic flies identified the *cis*-regulatory elements that are required for chorion gene amplification, termed amplification controlling

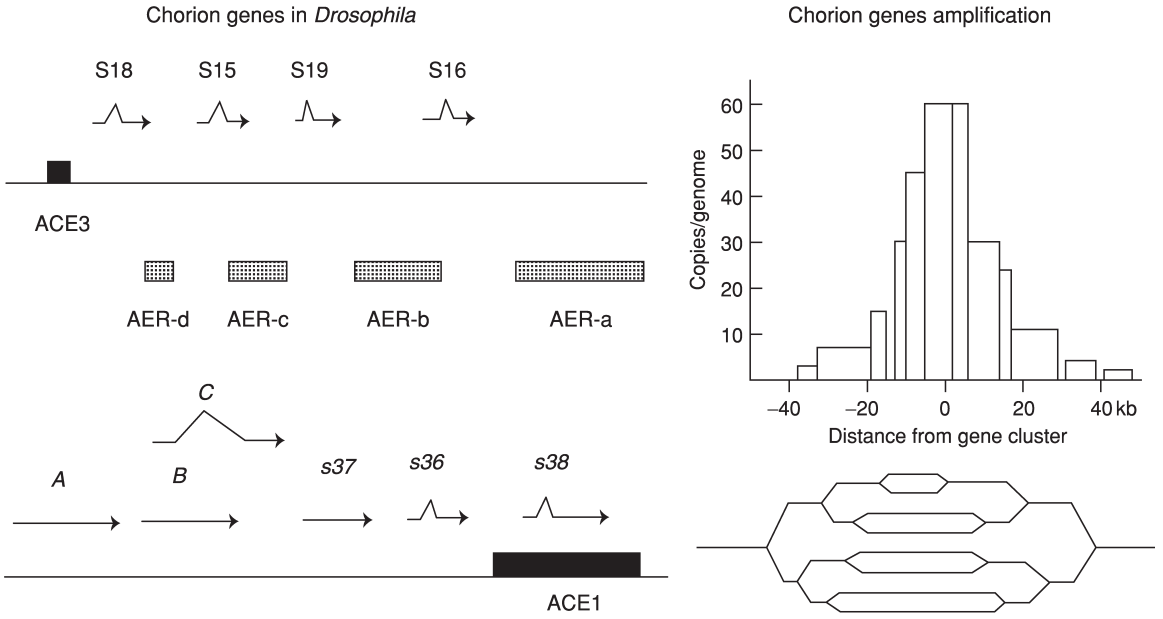


Figure 18 Chorion gene organization and mechanism of chorion gene amplification in *Drosophila*. Left panel: Structure of the chorion gene clusters. The third chromosome cluster above contains the genes for four major chorion proteins, s18, s15, s19, and s16. The X chromosome cluster below contains two major chorion genes, *s36* and *s38*, as well as several genes that encode minor chorion proteins (*A*, *B*, *C*, and *s37*). The positions of the transcripts are shown by the lines with arrows. Introns are indicated except for *A*, *B*, and *s37*, for which introns have not been mapped. The position of the amplification regulatory regions, AERs a–d (amplification enhancing regions a–d), and ACE1 (amplification control element 1), are also indicated. Right panel: Chorion gene amplification results from multiple rounds of reinitiation of DNA replication within the chorion gene cluster and progressive movement of replication forks to either side. Above is shown the level of amplification of DNA fragments at various distances from the origin of replication. Below is illustrated the ''onion-like'' structure of the nested bi-directional replication forks that are observed during chorion gene amplification. (Reprinted from Orr-Weaver, T.L., **1991**. *Drosophila* chorion genes: cracking the eggshell's secrets. *BioEssays 13*, 97–105.)

elements and amplification-enhancing regions (ACEs and AERs, respectively; **Figure 18**) (Kalfayan *et al*., 1985; Spradling *et al*., 1987; Delidakis and Kafatos, 1987; Swimmer *et al*., 1989; Lu *et al*., 2001). The origin of replication (Ori-β) within the third chromosomal cluster was also identified by two-dimensional gel electrophoresis (Delidakis and Kafatos, 1989; Heck and Spradling, 1990). Analysis of thin chorion mutants has revealed the involvement of components of the DNA replication complex, such as members of the origin recognition complex (ORC), Cdc7-Dbf4 kinase, double-parked protein (Dup)/Cdt1, and the minichromosome maintenance (MCM) protein complex (Landis *et al*., 1997; Landis and Tower, 1999; Claycomb *et al*., 2002; Schwed *et al*., 2002).

The initiation of chorion gene amplification is under cell cycle control. During their developmental career, two major switches in cell cycle control occur in the cells of the follicular epithelium. The follicular cells undergo mitotis until stage 6 of oogenesis, after which they undergo three endocycles (cycles consisting only of an S phase and a gap phase) and become polyploid (16N; Calvi *et al*., 1998; see **Chapter 1.2**). At the beginning of choriogenesis, the endocycles cease and DNA amplification is initiated at specific sites in the genome where the chorion gene clusters are located. The cyclin E cell cycle regulator plays a major role in orchestrating this transition: at the transition to choriogenesis, cyclin E switches from a cycling pattern of expression to continuous high expression in all follicle cell nuclei. It has been proposed that continuous high levels of cyclin E prevent general DNA replication in the follicular cell genome, while specific factors recruited at the chorion gene origins allow the latter to escape this negative replication control (Calvi *et al*., 1998). Factors that are involved in restricting or recruiting the replication machinery, specifically to the chorion origins of replication, are proposed to include the S-phase regulator E2F/DP/Rbf (Royzman *et al*., 1999; Cayirlioglu *et al*., 2001; Bosco *et al*., 2001) and a Myb (Myeloblastosis)-containing protein complex (Beall *et al*., 2002).

1.3.4.1.1.2. cis-Regulatory elements and factors that bind chorion gene promoter elements Promoter analysis in transgenic flies has been carried

out mainly for the *s15* and *s36* chorion genes, and *cis*-regulatory elements for temporal, spatial, and quantitative control have been delineated (Spradling, 1993; Kafatos *et al*., 1995). Short (less than 100–400 bp) segments of 5′-flanking DNA are sufficient to confer correct tissue-specific expression and precise temporal regulation of the transgenes (Mariani *et al*., 1988; Romano *et al*., 1988). *Cis*-acting regulatory elements, identified by mutational analysis, include an essential positive element, characterized by the sequence TCACGT that is conserved in all the chorion gene promoters, negative and positive elements that confer temporal (early or late) specificity, and elements defining spatially restricted expression (review: Orr-Weaver, 1991; Tolias *et al*., 1993; Mariani *et al*., 1996). In the latter case, the elements delineate expression in specific subregions of the epithelial surface, notably at the anterior and posterior poles and at the dorsal-anterior surface, where the dorsal appendages are formed (Parks and Spradling, 1987; Tolias and Kafatos, 1990).

Gel mobility shift assays were used to isolate putative *trans*-acting factors that bind and activate chorion gene regulatory regions (Kafatos *et al*., 1995). After a binding site was defined using nuclear extracts of choriogenic follicles, oligonucleotides encompassing the binding site were subsequently used to screen expression libraries and isolate binding proteins. This strategy resulted in the isolation of seven cDNAs that encode putative chorion gene transcription factors (chorion factors (CF) 1–7; Kafatos *et al*., 1995). Of these factors, CF1 and CF2 have been analyzed in more detail (Shea *et al*., 1990).

CF1 binds to the essential hexamer-element TCACGT. This factor was shown to correspond to the nuclear receptor USP (Henrich *et al*., 1990; Oro *et al*., 1990; Khoury-Christianson *et al*., 1992), the homolog of the vertebrate RXR receptor that functions as the heterodimer partner of the ecdysone receptor, EcR (Thomas *et al*., 1993; Yao *et al*., 1993). However, genetic analysis has shown that the involvement of CF1/USP in chorion gene expression may be more complicated than initially suggested by the DNA binding studies. Eggs laid by mutant flies that were usp^{-} in the germline cells (nurse cell oocyte complex) but wild-type (usp^{+}) in the somatic tissues (including follicular cells), showed a chorion defect, indicating that CF1/USP is required in the germline (but not in the follicular epithelium), in order to trigger the release of a signal that acts on the follicular cells and promotes chorion gene expression. *In situ* hybridization also showed that CF1/USP mRNA is abundant in the oocyte/nurse cell complexes and only barely detectable in follicular cells (Oro *et al*., 1992). The other possible roles of the EcR/USP complex during *Drosophila* oogenesis have already been discussed (Section 1.3.3.1.1.1.3).

CF2 was isolated by its property to bind the positive regulatory region that confers late temporal specificity to the *s15* promoter. It is a C_2H_2 zinc finger protein belonging to the class defined by the *Drosophila* regulatory gene *Krüppel* (*Kr*) and the mouse genes that encode the transcription factors MKR1 and MKR2 (Hsu *et al*., 1992). Genetic analysis, however, has shown that CF2 is involved in the dorsoventral patterning of the follicular epithelium (Hsu *et al*., 1996, Mantrova and Hsu, 1998). Upon activation of the epidermal growth factor receptor (EGFR) by the oocyte-derived Gurken (GRK) signal at the dorsal-anterior region of the follicular epithelium, CF2 is down-regulated by a posttranslational mechanism that involves phosphorylation by mitogen-activated protein kinase (MAPK) and subsequent cytoplasmic retention and degradation (Hsu *et al*., 1996, 2001; Mantrova and Hsu, 1998). Thus, CF2 protein expression becomes restricted to the ventral region of the follicular epithelium. One target gene of CF2 is *rhomboid* (*rho*), which encodes a transmembrane protein acting as a positive cofactor in EGFR signaling (Mantrova and Hsu, 1998). Because CF2 acts as a transcriptional repressor, degradation of CF2 in the dorsal cells of the follicular epithelium results in enhanced expression of RHO, which, in turn, results in the amplification of EGFR signaling levels in the dorsal-anterior follicular cells (Hsu *et al*., 1996; Mantrova and Hsu, 1998). The high levels of EGFR signaling are necessary for the formation of the dorsal appendages, the specialized chorion structures that mediate gas exchange between the environment and the embryo. In conclusion, however, as was the case with CF1, genetic analysis did not confirm a role for CF2 as a direct transcriptional regulator of the chorion genes.

Other chorion gene promoter binding factors that have been isolated and characterized include CF3, a zinc finger protein that interacts with the negative regulatory region in the *s15* promoter that is required for repression of its expression during early choriogenesis; three more factors (CF5–7) that bind the late activating region of the *s15* promoter; and CF4, which binds the early temporal element in the *s36* promoter (Orr-Weaver, 1991; Kafatos *et al*., 1995). CF5 has been shown to represent the *Drosophila* homolog of the human structure-specific recognition protein (SSRP), which belongs to the class of high-mobility-group (HMG) proteins (Hsu *et al*., 1993). HMG box-containing proteins are thought to function in processes such as DNA replication, DNA repair, and chromatin modeling, which may be involved in the regulation of transcription. The

localization of CF5 in the nucleolus and the observation that it binds single-stranded nucleic acid suggests that it may be involved in rRNA transcription through an RNA or single-stranded DNA binding mechanism (Hsu *et al*., 1993). Therefore, the role of factor CF5 in the regulation of chorion genes, as well as that of the other factors, CF3–4 and CF6–7, is not clear at present (Kafatos *et al*., 1995).

Recently, genetic analysis has indicated a role for the transcriptional regulator Tramtrack 69 (TTK69) in the regulation of chorion gene expression (French *et al*., 2003). Carriers of the female sterile allele of *tramtrack* (*ttk*) called *twin peaks* (ttk^{twk}), which affects the TTK69 transcription factor, are characterized by thin eggshells, presumably due to low levels of accumulation of mRNAs of the major chorion genes. However, the defects due to loss of TTK69 are considered to be indirect, because TTK proteins are known to function as transcriptional repressors, and no binding sites could be identified within 1 kb of any of the major chorion genes (French *et al*., 2003). It is, therefore, likely that TTK69 regulates the expression of factors upstream of the chorion genes rather than the chorion genes themselves. Besides influencing general chorion gene expression, TTK69 also has an independent genetic function in the formation of the dorsal appendages of the eggshell (French *et al*., 2003; see also below).

1.3.4.1.1.3. Signaling pathways Formation of the anterior and dorsal structures in the eggshell, such as the operculum, the micropyle, and the dorsal appendages, are subject to multiple signaling pathways (see Section 1.3.3.6). What will be discussed briefly here are transcription factors involved in the execution of the program of the formation of the dorsal appendages, two structures at the dorsal-anterior end of the eggshell that are responsible for gas exchange.

Signals that stimulate the EGFR in the follicular cells originally emanate from the Gurken (GRK, a TGFβ homolog) signal produced by the oocyte nucleus that is located at the dorsal-anterior end of the oocyte. Positive and negative feedback loops of signaling at the dorsal-anterior surface ultimately result in two patches of follicular cells, where high levels of EGFR signaling (Ras and MAPK activity) occur. These two patches of follicular cells are destined to form the dorsal appendages of the eggshell (Wasserman and Freeman, 1998; reviews: Stevens, 1998; Van Buskirk and Schüpbach, 1999).

Dorsal appendage formation can be divided in two stages; first, an initiation phase, during which the two groups of dorsal-anterior follicular cells move outward from the rest of the follicular epithelium and form a short tube overlying the junction between oocyte and nurse cells (stages 10B to 12 of oogenesis); and second, an elongation phase, during which the two tubes extend to their full lengths (French *et al*., 2003). The transcription factors Broad-Complex (BR-C), Pointed (PNT), and CF2 seem to have a role during the initiation phase of dorsal appendage formation.

Broad-Complex is a transcription factor characterized by C-terminally located C_2H_2 zinc fingers and an N-terminal Broad-Complex/Tramtrack/Bric-à-Brac (BTB) or poxvirus and zinc finger (POZ) domain, which appears to be involved in protein–protein interactions (DiBello *et al*., 1991; Collins *et al*., 2001; see also **Chapter 1.8**). The Z1 isoform of BR-C becomes expressed in the patches of follicular cells destined to form the dorsal appendages, and may be an upstream regulator of this process (Huang and Orr, 1992; Deng and Bownes, 1997).

Pointed is an ETS domain-containing transcription factor that is induced at the stage, when the highest levels of EGFR signaling occur. The expression of PNT results in a downregulation of the EGFR pathway, possibly through the induction of the expression of Argos (AOS), a putative extracellular inhibitor of EGFR (Schweitzer *et al*., 1995; Morimoto *et al*., 1996). The result of the action of PNT is the splitting of the original EGFR signaling area into two symmetrical patches across the dorsal midline of the egg, which will generate the two dorsal appendages.

As already mentioned earlier, CF2 is a transcriptional repressor whose expression is downregulated by EGFR signaling. Downregulation of CF2 results in the induction of RHO expression, which is involved in the amplification of the EGFR signal (Hsu *et al*., 1996; Mantrova and Hsu, 1998).

Finally, the Jun N-terminal kinase (JNK) cascade is involved in the correct execution of the extension phase of dorsal appendage formation (Dequier *et al*., 2001; Suzanne *et al*., 2001; French *et al*., 2003). Members of the JNK cascade include the *Drosophila* Jun N-terminal kinase kinase (DJNKK) or Hemipterous (HEP), DJNK or Basket (BSK), the transcription factors Djun and Dfos, and the JNK phosphatase or Puckered (PUC). In addition, the TTK69 transcriptional repressor (see above) also acts at the extension phase of dorsal appendage formation. Finally, the JNK signaling cascade is also required in the process of micropyle formation (Suzanne *et al*., 2001).

1.3.4.1.2. Lepidopteran insects The eggshell of lepidopteran insects constitutes a tripartite structure, consisting of the inner vitelline membrane, the chorion, and a very thin outer sieve layer (**Figure 17**;

Kafatos *et al*., 1977). The thickness of the vitelline membrane usually is $<2\,\mu m$, while the chorion can vary, depending on the species, from $<1\,\mu m$ to $>50\,\mu m$ (Regier and Vlachos, 1988; Leclerc and Regier, 1993). Two regions of the chorion are easily discernible, an inner trabecular layer and a much thicker overlaying lamellar chorion (**Figure 17**). Because the lamellar zone consists of helicoidally arranged planes of fibers, it is referred to as a "helicoidal lamellar chorion" (Mazur *et al*., 1982; Regier *et al*., 1995).

The helicoidal lamellar chorion structure is restricted to ditrysian lepidopteran insects. It is absent from the other insect orders, including the sister order of the Lepidoptera, the Trichoptera (Regier *et al*., 1995). Helicoidal lamellar chorion structures and protein compositions have been studied in detail in the superfamilies of Bombycoidea (mainly *A. polyphemus* and *B. mori*), Sphingoidea (*M. sexta*), and Noctuidea (*Lymantria dispar*) (Regier and Vlachos, 1988; Leclerc and Regier, 1993; Regier *et al*., 1995).

In the superfamily of Bombycoidea, the lamellae in the helicoidal lamellar chorion can number more than 100, and they are assembled by chorion proteins that can be grouped into two branches, α and β, and that belong to six families (ErA and ErB, A and B, HcA and HcB) (Regier *et al*., 1980, 1982; reviews: Kafatos *et al*., 1995; Eickbush and Izzo, 1995; see also Section 1.3.4.1.3 below). Other substructures of the chorion, the trabecular layer at the inner surface and the specialized structures at the external surface, generally contribute to no more than 5% of the mass of the chorion and contain unique subsets of proteins.

The formation of the helicoidal lamellar chorion is proposed to occur in four temporally discrete steps, framework formation (small number of lamellae are deposited), expansion (thickening of existing lamellae), densification (thickening of fibers within lamellae), and surface sculpturing (deposition of additional lamellae that can be sculpted into surface structures, e.g., the aeropyle crowns of *A. polyphemus* eggs; Regier *et al*., 1982; Mazur *et al*., 1989; Leclerc and Regier, 1993).

The different steps can be correlated with the expression of different types of chorion proteins that are synthesized during different periods in choriogenesis. Thus, in *B. mori*, ErA and ErB proteins are produced during framework formation, A and B proteins are secreted during the expansion and densification processes, and the formation of specialized outer lamellae is dependent on HcA and B proteins (see Section 1.3.4.1.3 below). The evolution of the chorion structure within ditrysian lepidopteran insects is proposed to have occurred by shifts in the timing and the intensity of protein synthesis in the four steps of assembly of the helicoidal lamellar chorion (Regier *et al*., 1995).

1.3.4.1.3. *Bombyx mori* The chorion or eggshell of *B. mori* (**Figure 17**) is an elaborate structure consisting of more than 100 polypeptides that are produced and secreted by the follicular cells during their terminal differentiation (Nadel *et al*., 1980; Iatrou *et al*., 1982). The chorion polypeptides are of low molecular weight (10–25 kDa) and have a tripartite structure consisting of a highly conserved central domain that is class-specific (A or B, see below), flanked by more variable left and right arms (Tsitilou *et al*., 1983). Sequence analysis reveals that chorion genes belong to a single gene superfamily that can be divided into two branches, α and β (A-type and B-type members). Each branch can be subdivided into three families or ErA/ErB, A/B, and HcA/HcB (Lecanidou *et al*., 1986).

Choriogenesis in *Bombyx* occurs in four broad developmental periods. Thus, early choriogenesis is characterized by the production of the ErA and ErB proteins (stages +1 to +8 in an ovariole with a choriogenic segment of 40 follicles). Most A and B proteins are synthesized during the early-middle and late-middle periods of choriogenesis (stages +6 to +24 and +10 to +28, respectively). Finally, late choriogenesis (stages +26 to +35) involves the production of high-cysteine (Hc) proteins HcA and HcB (Bock *et al*., 1986; Spoerel *et al*., 1986).

The chorion proteins that are produced during different developmental periods have specialized functions. Early proteins form an initial scaffold, into which middle proteins intercalate to add mass and density, while late (high-cysteine) proteins percolate throughout the previously deposited lamellae to crosslink the whole chorion and, in addition, form the outer lamellate crust (Mazur *et al*., 1982; Regier *et al*., 1982, 1995).

Classical genetic analysis, using protein electrophoretic variants as genetic markers, revealed that chorion genes are organized in two clusters on chromosome 2, Ch1–2, and Ch3 (**Figure 19**), that are separated from each other by 3–4 map units (Goldsmith, 1989). The organization of the chorion genes in two clusters was confirmed after isolation of overlapping chromosomal DNA clones, corresponding to the chorion locus, from genomic libraries of *Bombyx* (**Figure 19**). From cluster Ch1–2, two contiguous chromosomal DNA segments with sizes of 270 kb and 50 kb were isolated. The 270 kb fragment has a threefold organization, in which a central 140 kb region that contains the late Hc chorion genes, is flanked by segments that contain chorion genes of early-middle and late-middle specificity

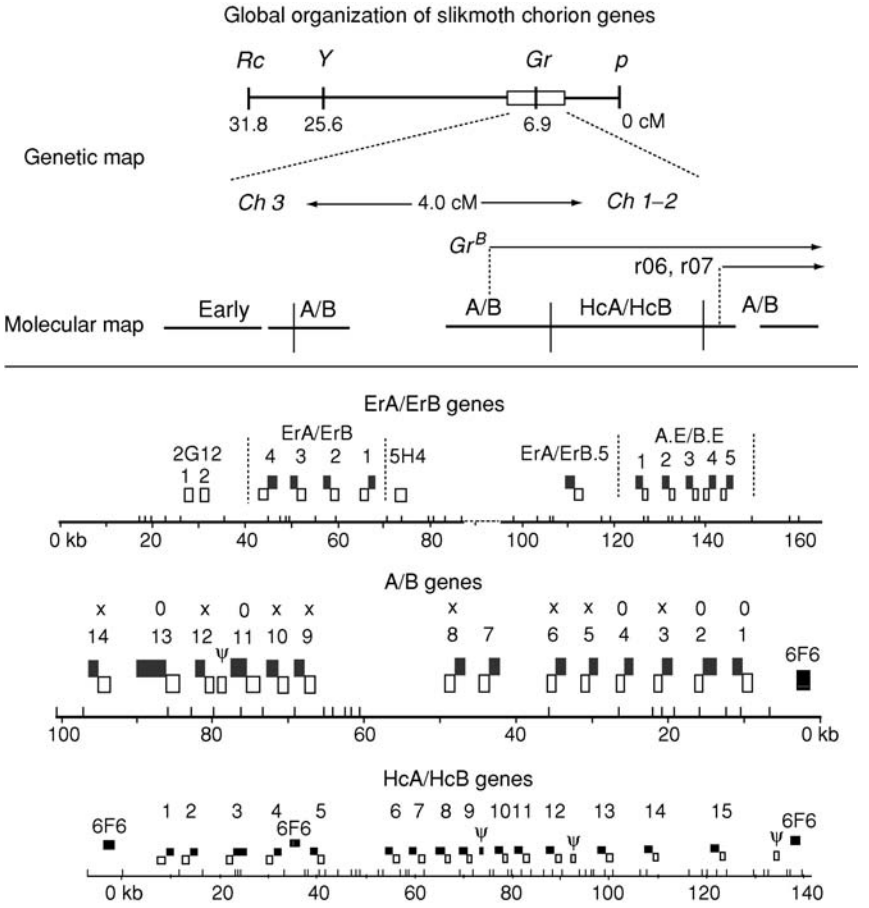


Figure 19 Chorion gene organization in *Bombyx*. Top panel: A portion of the genetic map of chromosome 2 is shown, with the genetic distances in centiMorgans (cM), for the following markers: *p*, plain larval markings; *Gr*, gray eggs; *Y*, yellow hemolymph and cocoons; and *Rc*, rusty cocoon color. The structural genes of the chorion proteins have been mapped to the gene complexes *Ch1–2* and *Ch3* (Goldsmith, 1989). Below the genetic map an overview of the molecular map is shown: the thick horizontal lines correspond to chromosomal segments from *Ch1–2* and *Ch3* that have been recovered as series of overlapping recombinant clones. The major families of chorion genes (early, A, B, HcA, and HcB) present on each cloned segment are indicated. Vertical lines on these chromosomal segments correspond to the boundaries between these gene families. An additional 80 kb of chromosomal DNA (not shown in the figure) has been recovered as a series of unlinked genomic clones containing A and B genes. The orientation and distance of the cloned segments from *Ch3* relative to that of the segments from *Ch1–2* are unknown. The segments of the *Ch1–2* complex deleted by the mutation, Gr^B, or translocated in mutants r06 and r07, are shown by the arrows above the cloned segments. Bottom panel: Organization of the early chorion gene complex (upper), the A/B gene cluster immediately to the left of the HcA/B gene cluster (middle), and the HcA/B gene cluster (lower). Filled boxes are α genes (ErA, A.E, A, and HcA) while open boxes represent β genes (ErB, B.E, B, HcB, 5H4, and 2G12). Almost all α and β genes are paired, with the exception of the 5H4 and two 2G12 genes. Pseudogenes are indicated by Ψ and the unpaired 6F6 (β branch type) genes are indicated by stippled boxes. Numbers refer to distances in kb. (Compiled from Eickbush, T.H., Izzo, J.A., **1995**. Chorion genes: molecular models of evolution. In: Goldsmith, M.R., Wilkins, S. (Eds.), Molecular Model Systems in the Lepidoptera. Cambridge University Press, Cambridge, pp. 217–247.)

(A and B genes). The 50 kb fragment also contains genes of middle developmental specificity (Eickbush and Kafatos, 1982; Spoerel *et al*., 1989). From cluster Ch3, two segments of 77 kb and 25 kb were characterized, in which early and early-middle chorion genes are present (Hibner *et al*., 1991).

The clustered organization of chorion genes on the chromosome according to their developmental specificity is intriguing, since it suggests that the different clusters may represent gene assemblies subject to common regulatory control, e.g., chromatin domains that become accessible to transcription factors at specific developmental stages (Eickbush and Kafatos, 1982; Goldsmith, 1989). Alternatively, the clustering of the chorion genes may be coincidental; it has been amply documented that diversification in the chorion locus has occurred by multiple duplication, deletion, inversion, and gene conversion events that involved relatively small patches of chromosomal DNA (Iatrou *et al*., 1984; Spoerel *et al*., 1986; Xiong *et al*., 1988;

Hibner *et al*., 1991; review: Eickbush and Izzo, 1995). In addition, it can also be noted that three chorion genes with early developmental specificity (e.g., the 6F6 genes) are found interspersed in the Ch1–2 locus (Kravariti *et al*., 1995), suggesting that opening of the Ch1–2 locus should occur concomitantly with the Ch3 locus and, therefore, arguing against the regulation of early versus middle and late chorion gene expression at the level of the gene cluster.

As a rule, chorion protein-encoding genes are organized in the *Bombyx* genome as gene pairs (**Figure 19**). Each gene pair consists of an A-type and B-type genes (ErA/ErB, A/B, and HcA/HcB), that undergo transcription in opposite and divergent direction from a common short (300 ± 30 bp) promoter region (Iatrou *et al*., 1984; Spoerel *et al*., 1986; Hibner *et al*., 1988). Paired genes are coordinately expressed during choriogenesis (Spoerel *et al*., 1986). Furthermore, through the use of heterologous functional assays, it has been demonstrated that the short spacer region between the two transcripts is necessary and sufficient for correct sex-, tissue-, and stage-specific expression (Mitsialis and Kafatos, 1985). A limited number of cases deviating from this rule of gene organization in pairs were also found. These involved some genes encoding early chorion genes, found in both the Ch1–2 and Ch3 clusters, which exist as single transcription units (Hibner *et al*., 1988; Lecanidou and Rodakis, 1992; Kravariti *et al*., 1995).

High-cysteine chorion proteins, that are synthesized during late choriogenesis, appear to be unique to *B. mori* and are not found in other silkmoths (Nadel and Kafatos, 1980). The production of high-cysteine proteins is a specialized (recently acquired during evolution) function of the *Bombyx* follicular epithelium: these proteins probably provide the eggshells with greater hardness and reduced permeability, both necessary for viability over the long periods of winter diapause (Regier *et al*., 1995).

1.3.4.1.3.1.* cis-*Regulatory elements and factors that regulate chorion gene promoter elements

Analysis of chorion gene expression in moths has been carried out using two different strategies. First, lepidopteran chorion promoters were cloned upstream of reporter cassettes and the activities of the chorion promoter–reporter cassettes and their mutant derivatives were assayed in transgenic *Drosophila* flies. Second, oligonucleotides encompassing chorion promoter segments were used in gel retardation assays to identify binding elements; the identification of binding sites subsequently allowed isolation of corresponding transcription factors or DNA binding proteins by homology cloning or screening of expression libraries (review: Kafatos *et al*., 1995).

When a genomic fragment containing the early-middle gene pair *A/B.L12* was introduced into the genome of *D. melanogaster* by P-element transformation, the silkmoth genes were expressed with correct sex, tissue, and cell specificity (Mitsialis and Kafatos, 1985). Thus, *A* and *B* genes were expressed coordinately in *Drosophila*, as in *Bombyx*, and their expression patterns resembled that of the endogenous *Drosophila* late gene *s15*. Comparable results were obtained with constructs, in which the common promoter of the gene pair (300 ± 30 bp) was placed upstream of reporter genes, encoding chloramphenicol acetyl transferase (CAT) or β-galactosidase, in either orientation (Mitsialis *et al*., 1987). It should be noted, however, that the temporal specificity was not strictly conserved between *Bombyx* and *Drosophila*, because the *A/B.L12* promoter, which is an early-middle promoter in *Bombyx*, behaved as a late promoter in *Drosophila* (Mitsialis *et al*., 1989). Opposite shifts in temporal expression (from late to early), after introduction into the *Drosophila* genome, were also observed for chorion promoters from the moths *A. polyphemus* and *A. pernyi* (Mitsialis *et al*., 1989).

The *cis*-regulatory elements of the bi-directional promoter of the *A/B.L12* chorion genes were defined after deletion and linker scanning mutagenesis in combination with P element-mediated genomic transformation of *Drosophila* (Mitsialis *et al*., 1987, 1989; Spoerel *et al*., 1993). Three important regulatory regions were detected, when mutant promoters were used in the heterologous functional assays. The regions were located asymmetrically in the promoters, and found to function in an orientation-independent manner and to have similar effects on the expression of both the A and the B genes in the pair. Thus, a TCACGT hexamer-element, located 60 bp upstream of the transcription start site of the *B* gene and found in the promoters of all silkmoth and *Drosophila* chorion genes, except the *ErA/B* genes of *Bombyx* (Hibner *et al*., 1991; Kafatos *et al*., 1995), was found to be absolutely necessary for expression; disruption of this element abolished expression of both the *A* and the *B* genes. Second, mutations in region A1, a 35 bp sequence located 185–220 bp upstream of the transcription start site of the *B* gene, resulted in a decrease of expression levels and alterations in temporal specificity. The complex behavior of this region could best be explained in terms of it having three distinct functional properties, acting as a general activator, an early repressor, and a late activator. Lastly, region A2, a 35 bp sequence located 140–175 bp upstream

of the transcription start site of the *B* gene, was shown to have a general activator function; in contrast to region A1, mutations in this region did not result in altered temporal expression.

To isolate *trans*-acting factors involved in the regulation of chorion gene expression, gel retardation assays, using regions of the chorion promoters as probes, were first carried out to define DNA elements that interact with proteins from follicular cell nuclear extracts. In a few cases, the sequence features of the element allowed an accurate prediction of the type of transcription factor capable of binding the element (Skeiky and Iatrou, 1991; Sourmeli *et al*., 2003). In other cases, oligonucleotides encompassing the elements were used to screen expression libaries from choriogenic follicles to isolate interacting factors (Kafatos *et al*., 1995). This strategy was used in order to identify binding factors in early, early-middle, and late chorion gene promoters (Skeiky and Iatrou, 1991; Kafatos *et al*., 1995; Sourmeli *et al*., 2003).

1.3.4.1.3.2. Early chorion gene regulators Band-shift assays, in combination with recognition by specific antibodies, have revealed that a CAAT/enhancer binding protein (C/EBP)-like transcription factor may be an important regulator of early chorion gene expression (Sourmeli *et al*., 2003). However, although C/EBP binding sites are more prevalent in early chorion gene promoters, such sites also occur in the chorion gene promoters of all developmental specificities. The C/EBP-like binding activity is also pronounced in nuclear extracts from early to middle choriogenic follicles, while its expression is reduced in late follicles. Thus, while the data indicate a predominant (positive) role for the C/EBP-like factor during the early stages of choriogenesis, other roles during the later stages of choriogenesis are also likely for this factor. It has been hypothesized that the C/EBP-like factor forms stage-specific complexes with other factors (e.g., the BmGATAβ factor, see below) during choriogenesis to direct stage-specific patterns of chorion gene expression (Sourmeli *et al*., 2003).

1.3.4.1.3.3. Early-middle chorion gene regulators The strategy of screening a choriogenic follicular library with probes corresponding to binding elements in the early-middle chorion gene promoter has resulted in the isolation of five classes of cDNAs, BmMCBP1–5, which encode putative regulatory factors (Kafatos *et al*., 1995). Of these factors, BmMCBP1–4 were found to bind the temporal regulatory region A1 (see previous section), while BmMCBP5 bound immediately upstream of the general activator region A2.

BmMCBP2, BmMCBP3, and BmMCBP5 each possess HMG-box (nucleic acid binding) regions; in addition, BmMCBP2 is characterized by a cysteine-rich region that resembles the zinc binding domain of the Trithorax (TRX) factor of *Drosophila*. BmMCBP4 has a central region that is homologous to the yeast SNF2 transcriptional activator. Finally, BmMCBP1 does not show significant homology to any transcription factors or other DNA binding proteins (Kafatos *et al*., 1995).

In *Drosophila*, the CF1 factor was isolated by its capacity to bind the essential TCACGT element in chorion gene promoters (Shea *et al*., 1990). Since the TCACGT element is also a prominent feature of all but the early *Bombyx* chorion gene promoters, it may be bound by the *Bombyx* homolog of CF1, BmCF1. BmCF1, which corresponds to the *Bombyx* homolog of USP, has been cloned (Tzertzinis *et al*., 1994) and the expression pattern of its mRNA was studied during follicle development (Swevers *et al*., 1995). The mRNA for BmCF1/USP is abundant in vitellogenic follicles, but its expression is down-regulated at the beginning of choriogenesis. Because data regarding the accumulation of the BmCF1/USP protein during oogenesis have not been reported, the role of BmCF1/USP in the control of chorion gene expression remains undetermined.

1.3.4.1.3.4. Late chorion gene regulators Gel retardation analysis using nuclear extracts of follicular cells resulted in the definition of two important binding sites in the common promoter region of one of the late gene pairs, HcA/B.12 (Skeiky and Iatrou, 1991). The DNA-binding proteins that bind these sites were designated *Bombyx* chorion factor I (BCFI; not to be confused with BmCF1/USP), and BCFII. BCFI has been considered to be the major determinant of late chorion gene expression, since binding of this factor to the late promoter region is prerequisite to the binding of BCFII and the formation of higher order complexes on the late promoters and, presumably, transcriptional activation (Skeiky and Iatrou, 1991). Both BCFI and BCFII factors have been cloned: BCFI corresponds to the BmGATAβ protein, a member of the GATA family of zinc finger motif-containing transcription factors (Drevet *et al*., 1994), while BCFII has been shown recently to correspond to a C/EBP-like transcription factor (Sourmeli, 2004).

BmGATAβ transcription is initiated during late vitellogenesis (stage "−6"), consistent with the postulated role of the encoded protein as a major regulator of choriogenesis. Regulation of *BmGATAβ* gene expression is complex and occurs both post-transcriptionally and posttranslationally (Skeiky *et al*., 1994; Drevet *et al*., 1995) (**Figure 20**). Three

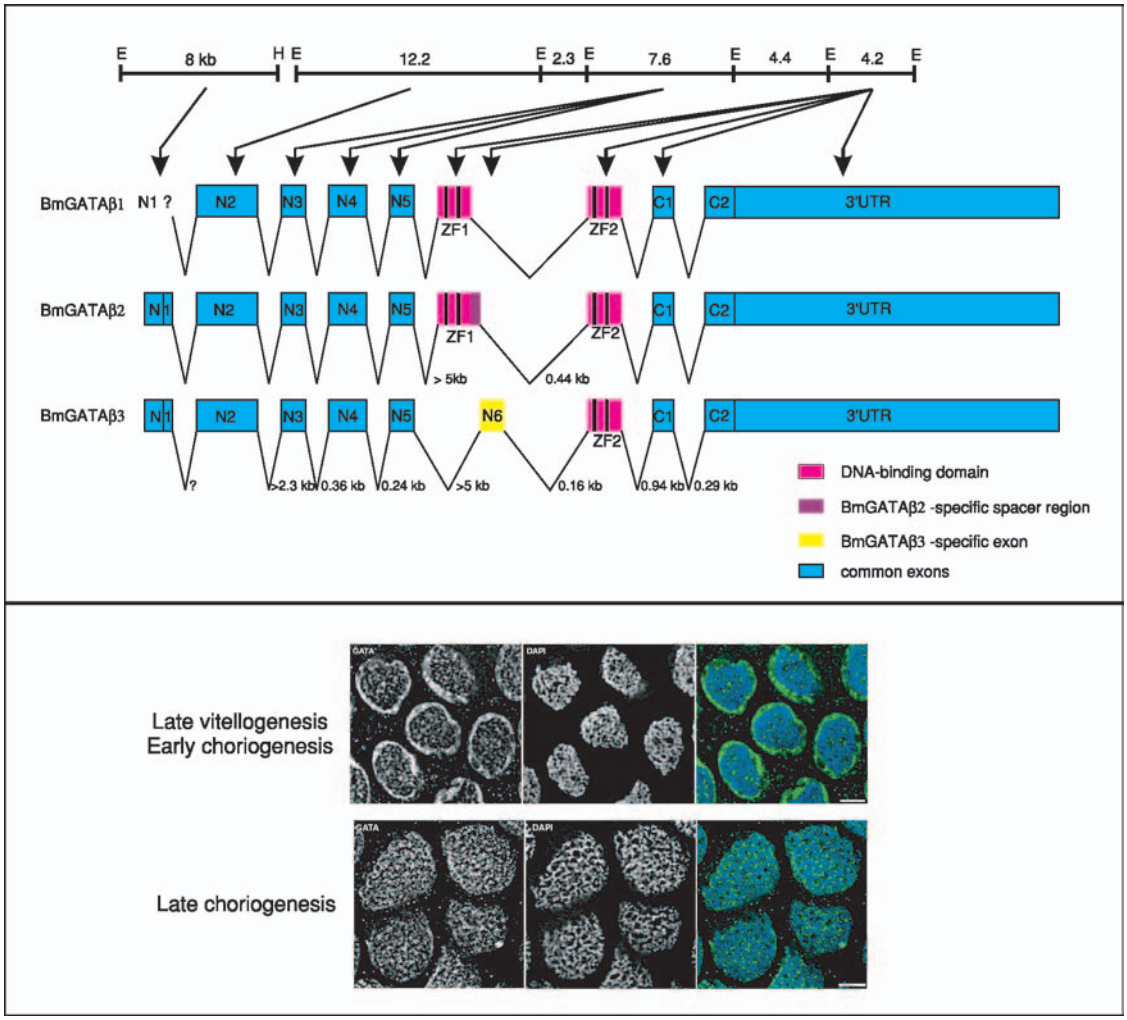


Figure 20 BmGATAβ transcription factors in *Bombyx mori*: structural features and subcellular localization in the cells of the follicular epithelium. Upper panel: Structure of the BmGATAβ gene and of the three major mRNA isoforms (β1, β2, and β3). The BmGATAβ gene consists of 10 exons, of which 9 exons could be located in a single contiguous DNA fragment that was assembled from overlapping genomic clones. The 10th exon, containing the N-terminal part of the β2 and β3 isoforms, belongs to a genomic clone that is not contiguous with the other genomic clones. E and H correspond to *Eco*RI and *Hin*dIII restriction sites, respectively, and the sizes of restriction fragments are indicated in kb. The positions of the exons of the BmGATAβ isoforms in the genomic DNA fragments are also indicated. The two exons that correspond to the N-terminal and C-terminal zinc fingers of the DNA-binding domain are in pink while the exons encoding protein regions N-terminal or C-terminal to the DNA-binding domain (N1-5 and C1-2, respectively) are in blue. In the BmGATAβ3 isoform, the exon of the first Zn finger is replaced by a unique sequence that is encoded by exon N6 (in yellow). Note that BmGATAβ1 and β2 isoforms differ by the spacing between the two zinc fingers (purple) and that the N-terminus of BmGATAβ1 is unknown. If known, the sizes of the introns that separate the different exons are indicated. Lower panel: Immunocytochemical localization of BmGATAβ protein in the follicular epithelial cells of ovarian follicles (Lunke, 2000). Shown is staining of follicular epithelia from late vitellogenic/early choriogenic follicles (top) and of follicular epithelia from late choriogenic follicles (bottom). Epithelia were treated with BmGATAβ antibody to localize BmGATAβ protein (left) or DAPI to stain nuclei (middle). At the right is shown an overlay of the left and middle images. Note that in late vitellogenic and early choriogenic follicles, BmGATAβ protein has both nuclear and perinuclear localization. In late follicles, however, BmGATAβ staining is exclusively nuclear. (Lower panel: Reprinted with permission from Swevers, L., Iatrou, K., **2003**. The ecdysone regulatory cascade and ovarian development in lepidopteran insects: insights from the silkmoth paradigm. *Insect Biochem. Mol. Biol. 33*, 1285–1297; © Elsevier.)

major BmGATAβ mRNA isoforms are generated that differ in the organization of their DNA binding domains: BmGATAβ1 and BmGATAβ2 differ by the spacing between their zinc fingers while BmGATAβ3 has the N-terminal zinc finger replaced by a new domain (Drevet *et al*., 1995). Because the zinc fingers of GATA transcription factors can have distinct functions in DNA binding and protein interaction (Omichinsky *et al*., 1993; Whyatt *et al*., 1993; Fox *et al*., 1999), the different isoforms are probably involved in the regulation of different sets of target genes. BmGATAβ2 seems to be the most

likely candidate to represent BCFI and regulate late chorion gene expression, because its expression is gonad-specific and its transcripts accumulate in ovarian follicular cells preferentially during late choriogenesis. By contrast, BmGATAβ1 and β3 have more ubiquitous expression patterns (Drevet *et al*., 1995), and may thus regulate gene expression in many different cell types.

The function of BCFI was also found to be regulated by posttranslational modification. Bandshift assays, using cytoplasmic and nuclear extracts of follicular cells, showed that BCFI remains predominantly in the cytoplasm of the follicular cells during early and middle choriogenesis, while during late choriogenesis, the GATA element (the late chorion promoter binding sequence for BCFI) binding activity is more concentrated in nuclear extracts (Skeiky *et al*., 1994). Furthermore, the distribution between cytoplasm and nucleus and the DNA binding specificity of BCFI were shown to be dependent of the phosphorylation status of this factor. However, immunocytochemistry experiments using an antibody that recognizes all BmGATAβ isoforms revealed that the BmGATAβ protein is present in the nuclei of follicular cells at all stages of choriogenesis, although cytoplasmic staining could also be observed in the cytoplasm of follicular cells of early and middle choriogenic follicles (**Figure 20**; Lunke, 2000; Swevers and Iatrou, 2003). A possible explanation may be that, of the existing multiple BmGATAβ isoforms, only one corresponds to BCFI and is involved in late chorion gene expression. While BCFI undergoes stage-specific nucleocytoplasmic shuttling, other isoforms may have a constitutive nuclear localization (Lunke, 2000) and be involved in the regulation of the expression of other target genes.

Because BmGATAβ binding sites (GATA elements) and C/EBP binding elements are present in all chorion gene promoters, and variable forms of the BmGATAβ and C/EBP-like factors accumulate in the nuclei of the cells of the follicular epithelium with different temporal specificities, it has been also proposed that the regulation of chorion gene batteries of different temporal specificities is achieved by the accumulation of different types of heteromeric complexes that contain stage-specific combinations of the C/EBP-like and BmGATAβ factors (Sourmeli *et al*., 2003). A complete understanding of the precise relationships among the various BmGATAβ isoforms, C/EBP proteins, and BCFI and BCFII-type factors, will require the availability and use of factor (and isoform)-specific antibodies in immunocytochemistry and gel retardation assays.

1.3.5. Ovulation and Egg Activation

Completion of choriogenesis is followed by ovulation. During ovulation, the layer of follicle cells is removed from the mature egg. The mature egg is subsequently squeezed out of the ovary into the lateral oviduct (Bloch Qazi *et al*., 2003).

In *Drosophila* and other insects, ovulation is stimulated by mating and, specifically by sperm and some male accessory gland proteins (Heifetz *et al*., 2000, 2001). Through a positive feedback mechanism, increased ovulation results in the stimulation of oogenic progression of follicles. The coupling of both processes ensures that high numbers of mature oocytes are produced and high numbers of fertilized eggs are deposited (Spradling, 1993).

After ovulation, egg activation is initiated, when the egg undergoes hydration in the oviduct (Heifetz *et al*., 2001). Features of egg activation include reinitiation of meiotic divisions (relief of arrest at metaphase I), impermeabilization of the egg coverings to small solutes (through crosslinking of the vitelline membrane and chorion) and initiation of translation (Page and Orr-Weaver, 1997; Chen *et al*., 2000; Bloch Qazi *et al*., 2003). All these changes will prepare the egg for subsequent embryogenesis, should fertilization occur in the uterus (Bloch Qazi *et al*., 2003). In *Drosophila* and many other insects, therefore, egg activation occurs independently of fertilization.

While the mature egg is activated in the oviduct, little is known about the fate of the follicular epithelium that was removed from the mature egg at ovulation. Most likely, these cells undergo necrosis or apoptosis and their remnants are removed by phagocytosis.

1.3.6. Conclusions and Perspectives

From the small number of insects in which oogenesis has been studied in sufficient detail, it is clear that considerable divergence exists in the regulation of oogenesis between different insects, e.g., the variety in the hormonal mechanisms that are involved in the control of vitellogenesis (Section 1.3.3.1). In some aspects, however, remarkable conservation can occur, as illustrated by the fact that lepidopteran chorion genes can become expressed with correct temporal and spatial specificity in transgenic *Drosophila* (Section 1.3.4.1.1.2). Presumably, the evidence related to the variety of control mechanisms that operate during insect oogenesis will increase even more, should oogenesis be studied in detail in more insect species. To have a more thorough understanding of the process of oogenesis in insects, many more models are therefore needed.

In this regard, it should be also noted that the insects that have been studied in detail thus far belong mainly to two orders, the Lepidoptera and the Diptera. Moreover, from these model insects, by far the greatest wealth of information is derived from the fruit fly, *D. melanogaster*. This is due to the fact that the methods of genetic analysis are very advanced in the fly and that *Drosophila* has functioned as a model not only for insects but, also, for many other multicellular eukaryotes, including humans.

Because methods of genetic analysis have not been used in other insects until very recently, data regarding the *in vivo* function of oogenesis-related factors in other insect species are lacking. Thus, for *B. mori*, only one report exists in the literature regarding the temporal specificity of chorion gene promoters after introduction of artificial promoter-reporter constructs into the cells of the follicular epithelia by the biolistic method (Kravariti *et al.*, 2001). In this study, a small 5′-flanking region of the 6F6 early gene and the small intergenic region of the late HcA/B.12 gene pair were shown to be able to direct expression of the reporter gene in the follicular epithelia with correct temporal specificity.

Recently, however, genetic transformation of non-drosophilid insects by transposable elements has become possible in a number of other dipteran insects, including *A. aegypti*, and the lepidopteran *B. mori* (Kidwell and Wattam, 1998; Tamura *et al.*, 2000; Horn and Wimmer, 2000; Handler, 2001; Atkinson, 2002). In addition, a technique for gene targeting in the silkmoth has been developed through the use of baculovirus vectors (Yamao *et al.*, 1999). Pantropic retroviral vectors and sindbis viruses are also regarded as promising tools for foreign gene delivery in somatic insect tissues (Jordan *et al.*, 1998; Foy *et al.*, 2004). Therefore, the availability of genetic transformation techniques for additional model insects will allow the *in vivo* functional testing of the involvement of particular factors that were identified by *in vitro* methods, in oogenesis. As long as these types of experiments have not been carried out, the function of the identified factors (e.g., in *Aedes* or in *Bombyx*) remains speculative.

Another recent development is the application of the technique of RNA interference (RNAi) to knock down the function of genes in cells and tissues (Hannon, 2002). RNAi is based on the introduction into the cells of double-stranded RNA, which becomes processed by the endogenous cytoplasmic enzyme Dicer into small (20–22 nt) double-stranded RNA species known as "small interfering RNAs" (siRNAs). siRNAs subsequently become integrated into a multiprotein complex known as the RNA-induced silencing complex (RISC), where they function as guide RNAs to target homologous mRNAs for cleavage and degradation (Dykxhoorn *et al.*, 2003). The advantage of the technique of RNAi is that it can be easily applied to knock down gene expression and that no extensive knowledge of the genetic make-up of a particular organism is required. The technique of RNAi has already been applied successfully in several insect species (Marie *et al.*, 1999; Lam and Thummel, 2000; Quan *et al.*, 2002; Bettencourt *et al.*, 2002; Blandin *et al.*, 2002; Rajagopal *et al.*, 2002; Valdes *et al.*, 2003; Uhlirova *et al.*, 2003), therefore it holds great promise for the genetic analysis of gene function in insects, in general, including oogenesis.

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