Molecular Analysis of Ovary Specific cDNAs from Galleria Mellonella

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MOLECULAR ANALYSIS OF OVARY SPECIFIC cDNAS FROM *GALLERIA MELLONELLA*

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ABSTRACT

MOLECULAR ANALYSIS OF OVARY SPECIFIC cDNAS FROM*GALLERIA MELLONELLA*

To study the regulation of sex-specific gene expression during oogenesis, a vitellogenic ovariole cDNA library was constructed and differentially screened with ovary and male pharate pupal total cDNAs. Six classes of ovary specific clones were isolated. Clones, F11, F20 and N23 represent 1.1, 2.0 and 2.3 kb ovary specific transcripts. *In situ* hybridization and developmental Northern analysis showed that F11 and F20 transcripts are limited to vitellogenic follicle cells. N23 transcripts are present in the pre-vitellogenic and vitellogenic nurse cells, and they persist in choriogenic follicles and early embryos suggesting that N23 may represent a maternal message.

The 0.95 kb and1.6 kb cDNAs of F11 and F20, bear a single open reading frame and code for peptides of 286 and 504 amino acids, respectively. The 952 bp F11 cDNA, appears to contain the entire coding region including a translation initiation codon (4 - 6), a stop codon (862-864) and the poly A+ signal sequence (935-941). The deduced amino acids 18 to 28, of the F11 matched *Galleria* YP4 yolk polypeptide N-terminal amino acid sequence. The deduced amino acid sequence of F20 shows overall identity of 42 % to *B. mori* egg specific protein (ESP) and has a lipid binding domain (aa 271 to 281) found in mammalian lipases, *B. mori* ESP (aa 322 - 332) and *Drosophila* yolk proteins (YP1- aa 248 - 257). F20 has also a serine rich domain that can be phosphorylated and four potential N-glycosylation sites. These observations suggest that F20 may represent the follicle specific yolk polyptide-YP2 gene. Genomic Southern and PCR analyses suggest that *yp4* is a single copy gene without introns while F20 probably has introns and belongs to a small gene family.

F20 and YP4 are not expressed following *in vitro* culture but are expressed in ovariestransplanted into male larval hosts, suggesting that the developmental expression of the follicle specific yolk protein genes may depend on the interaction of the ovary with other pharate adult tissues.

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INTRODUCTION

Context and Objectives

Oogenesis in insects and other higher coelomates involves coordinated expression of sex specific genes not only in the ovary but also in extra ovarian somatic tissues. An understanding of the molecular basis and regulatory mechanisms underlying the oogenic events can be best appreciated by a molecular analysis of the structure and expression of these female specific genes.

In the course of oogenesis, the developing oocyte accumulates yolk proteins and is supplied with maternal messages for the eventual development of the embryo. The nutritional requirement of oviparous vertebrate and invertebrate embryos are supplied by yolk proteins, a class of glycosylated phospholipoproteins (see, Kunkel and Nordin, 1985; Wahli and Ryffel,1985). During vitellogenesis, vitellogenins (Vgs) the precursors of yolk proteins are typically synthesized in extra-ovarian tissues and secreted into the body fluids (blood in vertebrates; and hemolymph in insects) for uptake by developing oocytes (Telfer et al, 1981; see, Kunkel and Nordin, 1985). Generally, Vg undergoes various postranslational modifications such as glycosylation, phosphorylation and proteolytic cleavage prior to their secretion and selective uptake (Wang et al. 1983; Sharrock 1984; Dhadialla and Raikhel,1990). Finally, they are taken into the oocyte by endocytosis and accumulated in specialized vesicles to form yolk platelets (see; Kunkel and Nordin,1985; Raikhel and Dhadialla,1992).

Sex and tissue specificity of yolk protein expression has been reported for a wide variety of organisms including nematodes, sea urchins (Speith and Blumenthal,1985; Shyu et al,1986), several groups of insects (Bownes 1986; Wheeler and Kawooya,1990; see; Wahli, 1992), fish (Maitre et al, 1985), amphibians (Wahli and Ryffel,1985) and birds (Wang and Williams,1982). The yolk protein genes are usually expressed in extra ovarian tissues, such as the liver in chicken (Wang S-Y and Williams DL,1982) and *Xenopus*

(Wahli W and Ryffel GU,1985); the intestine in nematodes (Speith and Blumenthal, 1985) and sea urchins (Shyu et al,1987); the fat body cells (- a heterosynthetic organ comparable in function to vertebrate liver) in insects (see; Raikhel and Dhadialla, 1992).

However, in insect species, such as the higher dipteran *Drosophila* (Brennan et al, 1982) and certain lepidopterans such as *Bombyx mori* (Sato and Yamashita, 1989) and the pyralid moths to which *Galleria mellonella* (Shirk, 1987) belongs, ovarian follicle cells also synthesize yolk proteins. In the stable fly *Stomoxys*, all the yolk proteins were shown to be made in the follicle cells (Chen et al, 1987). While the three yolk protein genes (yp1, yp2 and yp3) of *Drosophila melanogaster* are expressed both in the fat body cells and the ovarian follicle cells (Garabedian et al, 1985, 1987), the fat body cells and the ovarian follicle cells in moths appear to express different sets of yolk protein genes (Ono *et al.*, 1975; Bast and Telfer, 1976, Telfer *et al.*, 1981; Shirk, 1987). The physiological factor(s) and molecular mechanisms regulating oogenesis and yolk protein genes in lepidopterans are not known. Yolk protein genes are expressed as a specific response to hormone: estrogen in vertebrates, juvenile hormone in some insects such as the locust, or ecdysone in some dipterans (Wang and Williams 1982; Wahli and Ryffel 1985; Bownes 1986; Gemmill et al,1986; Wyatt (1988). However in the moths, there is no evidence yet for a direct hormonal regulation of yolk protein gene expression. The isolation of a molecular probe for a yolk protein cDNA from *Galleria* reported here should allow us to elucidate the regulatory events leading to yolk protein gene expression during moth oogenesis.

Because yolk proteins constitute an obvious set of sex-specific gene products, my effort was concentrated on isolating a yolk protein cDNA and analyzing its expression and gene structure in *Galleria mellonella.* In *Galleria* there are four yolk polypeptides which are designated YP1 to YP4 in descending order of molecular weight (Shirk *et al.*, 1987; Bean *et al.*, 1988). While YP1 (159 kDa) and YP3 (44 kDa) are produced by the fat body, YP2 (74 kDa) and YP4 (37 kDa) are synthesized by the ovarioles (Shirk, 1987). The

advantage of the dual sites of synthesis of different yolk polypeptides in the moths (as opposed to dual sites for the same polypeptides in the higher dipterans) is not known.

Except for the cDNA and genomic sequences for the egg specific protein (ESP) from *B. mori* (Inagaki and Yamashita,1989; Sato and Yamashita, 1991) and the fat body specific microvitellogenin from *Manduca sexta* (Wang et al, 1988), no other yolk sequences are available for Lepidoptera. For a valid evaluation of evolutionary relationships of the yolk protein genes, sequence information from more species is necessary. The isolation and sequencing of *G. mellonella* follicle-specific yolk protein cDNAs should lead to the study of evolutionary history of yolk protein genes and possibly to the evolution and the significance of multiple sites for yolk protein synthesis in the moths and higher dipterans.

As a first step to identify physiological factor(s) involved in the regulation of oogenesis, and the elucidation of the molecular mechanisms underlying the expression of two different sets of yolk protein genes in two different tissues (fat body and ovary) in lepidopterans, and to establish the evolutionary relationship between ovarian follicle cell specific yolk protein genes in the moths and those of higher dipterans (*Drosophila* yps), I set as my goal to isolate a follicle cell specific yolk protein cDNA and analyze the expression and structure of the corresponding gene, in *Galleria mellonella.*

The research reported here includes, the isolation and characterization of three ovary specific cDNAs from the wax moth *Galleria mellonella.* Based on the developmental analysis, the cDNA clone N23, represents a nurse cell produced message present also in the embryo. Based on the sequence analysis, tissue and developmental stage specificity of expression, one of the ovary specific cDNAs, F20 cDNA is the putative YP2 encoding cDNA, while Ov11 is shown to code for the yolk polypeptide-YP4 of *Galleria.* Genomic Southern analysis, and PCR cloning of the YP4 genomic fragment, suggest that the YP4 gene is an intronless, single copy gene.

Ovary transplantation into male milieu and analysis of the expression of these genes suggest that any sex specific physiological factors are locally produced, by the ovarian tissue. In addition, an extra ovarian factor (other than ecdysone), most likely induced by a signal from the ovary may also be involved in regulating yolk protein production.

Background

Insect egg proteins are contributed by two tissues- the fat body and the ovary. In considering the sex limited gene expression, the two tissues exhibit different regulatory programs. Males do not have ovarioles. The fat body cells, which exhibit similar cytomorphology in both sexes, can express some genes e.g., those for yolk proteins (Vgs) and the so called female specific storage proteins or larval hemolymph proteins (SP1, LHP-81), in a sex-limited or sexually dimorphic manner (ie: in the females only; Bean and Silhacek, 1989). The expression of these genes may be regulated by hormones, and the developmental program may be altered by changes in the expression pattern of sex determination genes or by application of hormones, illustrated by the reversal of YP expression in adult tra-2^{ts}*Drosophila* by temperature shift (Belote and Baker, 1987) and by the inducibility of Vg genes in the male locust nymphs by JH (Locke et al, 1985; Wyatt, 1990) respectively. In this context it is interesting that no major male specific gene products have been described in the fat body. This probably reflects the lesser need for gametic storage for proteins in the male, mediated by a sex determination pathway which exerts positive control in the female, leaving the male in a default state.

In the ovary on the other hand, the tissue itself is sexually differentiated, forming part of the morphological gender phenotype. Here, while the transcription of specific genes may be under hormonal control, determination of their expressible state must be correlated with differentiation of the tissue. In some insects such as the higher dipterans

and a few lepidopteran species, the fat body and ovarioles exhibit temporally coordinated sex specific gene expression during vitellogenesis.

Life cycle and vitellogenesis

Holometabolous insects such as dipterans and lepidopterans undergo complete metamorphosis (from larva to pupa and then to adult), a sequence of developmental changes controlled by the interaction of two hormones, juvenile hormone (JH) and 20-hydroxy-ecdysone (20- HE). Pupae in which adult development has been initiated is referred to as pharate adult. The stage at which oogenesis occurs is correlated with the feeding habits of the adult insect. In insects which feed as adults and those with several reproductive cycles such as *Drosophila* (Bownes,1982a), *Aedes agypti* (Gemmill et al,1986), bees (Trenczek et al,1989), cockroach (Mundall et al,1983), and locust (Glinka and Triselva,1989) several batches of eggs are laid. Yolk protein synthesis and uptake (ie. vitellogenesis) occurs in the adult stage usually under hormonal control. Depending on species, ecdysteroids and/or juvenile hormone (JH) regulate vitellogenesis.

In *Galleria mellonella* and other related lepidopterans which do not feed as adults and have a short life span, vitellogenesis takes place in the pharate adult (pupae in which adult development has been initiated) stage. By the time the adult emerges from the cocoon, it contains fully developed eggs ready to be fertilized and laid. Vitellogenesis is more or less completed within the time frame of the hormonal regimen regulating adult development. During its life cycle, *Galleria* undergo complete metamorphosis from a feeding mobile larva (instars 1 to 7) to a quiescent non-feeding pupa. Vitellogenesis is included among the developmental changes associated with oogenesis and adult development. In the larval stage, the fat body cells synthesize the LHPs (LHP 74, 76 81 and 82) which are under the regulation of ecdysone and JH (Ray et al, 1987; Bean and Shirk, 1989; see: Kanost et al, 1990; Memmel et al, 1994). However, during vitellogenesis in the pharate adult female, the same fat body cells express vitellogenins which give rise to the yolk proteins YP1 and YP3. At the same time the ovarian follicle

cells produce two follicle specific yolk polypeptides -YP2 and YP4. In *Galleria*, the regulatory factors and molecular mechanism underlying the switch from synthesis of LHPs to Vgs synthesized in the fat body cells, and the temporal regulation of YP expression in the ovarian follicle cells are not known.

Types of ovarioles in insects

Three basic types of ovary organization are found in insects. They are 1) panoistic, 2) meroistic telotrophic and 3) meroistic polytrophic types. In the panoistic ovary, most of the RNA resources of oocytes are provided by synthetic activity of the oocyte nucleus itself. The developing oocytes of many animals including panoistic insects such as the *Acheta domesticus,* amplify their rDNA genes in order to produce a sufficient complement of ribosomes for the egg (Cave and Allen, 1974). Ancestral insects are believed to have panoistic ovaries and these have been retained by some orders (eg:- Orthoptera) while the meroistic ovary has evolved in others (eg:- Lepidoptera, Hymenoptera and Diptera).

These higher insects have evolved a highly effective mechanism for loading their unfertilized eggs with long-lived messenger RNAs, ribosomes, transfer RNAs and enzymes which are later utilized for synthesizing the proteins needed by the developing embryo. Polyploid nurse cells produce the required compounds and a system of trophic cords or ring canals develop, through which these products are exported to the oocyte. Nurse cells in *Bombyx mori* (lepidoptera), display high level of polyploidy. However, the fact that the proportion of the genome coding for rRNA is the same for silk gland (polyploid), for carcass (mostly diploid), for testis (diploid and haploid cells), and isolated ovarioles of early pupae and 10 day old pupae, indicates that rDNA is not selectively amplified in the nurse cells (Cave and Sixbey, 1976). The nurse cell cysts and the oocytes of the giant silkmoth, *Antheraea pernyi*, also contained the same proportion of rDNA as the somatic cells (Cave, 1978). The fact that the same proportion of DNA from different tissues hybridize to rDNA indicates that in the polytrophic ovary, polyploidization of the

entire nurse cell genome occurs without any further amplification of the rDNA. In *Drosophila melanogaster*, the increased rate of rRNA synthesis per egg chamber parallels the number of rRNA genes (Mermod et al, 1977). Fertilized eggs that lack sex chromosomes (the chromosomal sites of the ribosomal genes) can undergo 10-12 mitotic divisions before dying. This shows that protein synthesis during early embryogenesis occurs entirely on ribosomes provided by the nurse cells during oogenesis (von Borstel and Rekemeyer, 1958).

Nurse cells are either enclosed in each egg follicle in association with the oocyte as in polytrophic ovarioles, or are located separately in the tropharium as in telotrophic ovarioles. Polytrophic and telotrophic ovarioles differ only in the way the maternal ribosomes and RNAs are transported to the oocyte. Oocyte-nurse cell syncytia are formed in all insects characterized by meroistic polytrphic ovarioles (King and Aggarwal, 1965; Telfer, 1975) including dipterans and lepidopterans.

Formation of an egg follicle

The insect egg follicles develop within the ovary from a group of cells originating from germline (oocyte and nurse cells) and mesodermal somatic cells (follicle cells). Oogenesis is a cooperative process that involves interaction between follicle cells, nurse cells and the oocyte. Developmental changes in the oocyte and the associated cells have been studied in the dipteran, *Drosophila* (Koch and King, 1967; Mahowald, 1972) and the moths, *H. cecropia* and *B. mori* (King and Agarwal, 1965; Telfer, 1975; Yamauchi and Yoshitake,1984). Oogenesis has been divided into 14 stages in *Drosophila* and 12 stages in the moth *B. mori* based on electronmicroscopic (EM) analysis of successive stages of the developing oocyte. A series of developmental events in oogenesis lead to the organization of the primary oocyte, which is endowed with maternal gene products and nutrients to support embryogenesis. In a wide variety of insects primary oocytes are arrested in metaphase of the first meiotic division (Yamauchi and Yoshitake,1984).

Each of the paired ovaries in moths consist of four ovarioles, each of which contains a chain of follicles in progressively advanced stages of development. An apical, mitotically active area called the germarium contains in its most anterior region a few germ cells followed posteriorly by clusters of interconnected cells with two, four and eight cystocytes. After multiple divisions, the cluster of germ line stem cells start to produce oogonia. Each stem cell divides forming two daughter cells; the apical cell (towards the germarium) continues to behave as stem cell whereas the other functions as a cystoblast or the oogonium (Mahowald and Kambysellis, 1980, King and Bunning,1985). Each germarium contains a few oogonial cells. The cystoblasts divide mitotically, but the daughter cells (cystocytes) do not increase in size. In the lepidopteran moths, the cystoblast divides three times to give 8 cystocytes one of which differentiates into the oocyte while the rest give rise to the seven nurse cells (Telfer, 1975; Woodruff and Telfer, 1990). The follicle cells, which form a single layer of epithelium around the oocyte-nurse cell cluster, originate by multiple divisions from a mesodermal somatic cell line. The oocyte and its seven nurse cells are connected by intercellular bridges formed by incomplete cytokinesis at the time of cystocyte divisions. The first visible signs of nurse cells coincide with encapsidation of the cystocyte cluster by the monolayer of follicle cells to form the egg chamber or follicle. As the oocyte assumes a posterior position in the follicle, the follicular epithelium around the oocyte becomes columnar whereas those over the nurse cell remain cuboidal. The egg follicle in the moth consists of seven nurse cells and an oocyte surrounded by a single layer of follicle cells; the follicle is considered the functional developmental unit in oogenesis.

Developmental stages of egg follicles

In the moths, each ovariole includes a germarium and a vitellarium. The germ cells proliferate and follicles are formed at the apical region of the germarium. The vitellarium is where follicles undergo vitellogenesis (yolk protein synthesis and accumulation). Egg

follicles are arranged in a linear array and each represents a defined stage of oogenesis differing from that of its neighbor by a few hours of developmental distance (Telfer,1954; Zimowska,1991; Swevers and Iatrou, 1992). At the posterior end the four ovarioles of each side open into a lateral oviduct. During the larval stage, the four ovarioles develop within an ovarian capsule (Miya et al, 1970b). At the onset of the pre-vitellogenic stage, the capsule tears at its attachment point to the oviduct and the ovarioles emerge gradually by elongation into the abdominal cavity. Follicle development commences with the appearance of the oocyte-nurse cell complexes and terminates with the organization of the mature eggs. As described earlier, follicular development in the moths is divided into 12 stages based on morphological criteria.

Among the 12 stages (S1-S12) of follicle development in the moths, pre-vitellogenic stages (S1 to S3), vitellogenic stages (S5 to S10) and the chorionic stages (S11 to S12) are observable at the same time in a late pharate adult or adult ovariole. The mature ovarian egg (chorionated /S12) is liberated into the oviduct. Thus, depending on the time of dissection, each ovariole can provide egg follicles at various stages of oogenesis from pre-vitellogenic to vitellogenic to choriogenic stages. This facilitates the study of the molecular developmental changes that occur during the transition of follicles from one stage to another.

Function of nurse cells

Oocyte development is supported by the two types of ovarian, endopolyploidic accessory cells- the follicle cells and the nurse cells. They change their activities as oogenesis progresses from pre-vitellogenic to vitellogenic and then to post-vitellogenic and choriogenic stages (Mahowald,1972,Telfer,1975). The nurse cells are transcriptionally very active (Telfer,1975; King and Bunning, 1985) and provide the maternal RNAs, proteins and protein synthetic machinery to the oocyte, from the beginning of pre-vitellogenic stages to the end of vitellogenic stages. The oocyte chromosomes are at

pachytene stage during S1to S3 and enter metaphase I by S10, then becoming transcriptionally less active. At the end of vitellogenesis (by S9), the nurse cells complete the transfer of cytoplasm to the oocyte, close the cytoplasmic connections and disintegrate by S10. The maternal messages function in the early embryonic pattern formation, when they activate the developmental cascade of gene expression in a spatially specific manner, their roles have been extensively studied in *Drosophila* (see, Lehmann, 1988).

Function of follicle cells

Follicle cells have two major functions: 1) they form an epithelium around the oocyte and depending on stage of oogenesis, they allow or obstruct the transmission of yolk precursors from the hemolymph into the oocyte; 2) They express stage specific genes and contribute to yolk and egg shell formation.

As an insect egg follicle becomes vitellogenic, the oocyte must assemble sufficient cellular machinery and gain access to the necessary precursors to support the subsequent rapid accumulation of yolk proteins and formation of yolk spheres or yolk platelets (Telfer,1965; see, Engelman,1979; Telfer et al,1982; see, Kunkel and Nordin,1985). Insect oocytes assemble their protein yolk granules by receptor-mediated endocytosis of specific extracellular proteins (see, Raikhel and Dhadialla,1992). In pre-vitellogenic oocytes of the anautogenous mosquito, *Aedes aegypti,* the production of a specialized cortex containing numerous coated pits, vesicles, and endosomes in or near the brush border, is necessary to support the rapid uptake of yolk proteins following the onset of vitellogenesis after a blood meal (Raikhel and Lea, 1985). In order for the oocyte to gain access to vitellogenins produced in the fat body and transported to the ovary in the hemolymph, the follicular epithelium must develop patency due to the development of spaces between follicle cells (Pratt and Davey,1972). Patency, thus, facilitates protein diffusion from the hemolymph through the ovarian sheaths and between the follicle cells. The yolk precursors traverse these routes and bind to putative receptor molecules on the

surface of the oocyte. Endocytosis of bound proteins is effected by clathrin-coated vesicles, which after appropriate modification in the cortex of the oocyte, deliver their load of extracellular protein to the growing yolk spheres by membrane fusion (Anderson, 1964).

Vitellogenic egg proteins

A. Fat body specific proteins:-

A sex-limited "female protein" was first found in *H. cecropia* (silk moth) hemolymph and was shown to participate in yolk formation by Telfer (1954). The major source of this protein was identified as the fat body and the protein was named vitellogenin (Vg), the precursor of vitellin, or yolk protein. Vgs from a number of insects representing different orders are phosphoglycolipoproteins consisting of arge (120- 200 kD) and small (40-60 kD) size classes, all sharing somewhat similar amino acid composition (see; Kunkel and Nordin, 1985). *In vivo* , mature Vgs are generally the products of proteolytic processing; pulse labeling of fat body in Locust (Chen et al, 1978), *Leucophaea maderae* (Della Cioppa and Engelmann, 1987) and other insects shows incorporation of labeled amino acids into polypeptides of $\geq$ 200 kDa. In the mosquito, *Aedes aegyptii,* the processing steps have been traced from a preprovitellogenin of 224 kD through glycosylation, phosphorylation, sulfation, and secretion to mature vitellogenin with 200kD and 66 kD subunits (Dhadialla and Raikhel, 1990). Vg mRNAs of 6.3 - 6.5 kb have been seen in the fat body of *Locusta migratoria* (Chinzei et al., 1982) *Aedes agyptii* (Gemmill et al,1986) and some other species (see Harnish et al,1982) accounting for both large and small subunits. However in *B. mori*, the large and small subunits appear to be translated from separate mRNAs of appropriate size (Izumi and Tomino, 1983). Yolk proteins of higher diptera (suborder; Cyclorrhapha) are quite different from other insect vitellogenins. In *D. melanogaster*, the three yolk poly peptides of of 46, 45 and 44 kD, called YP1, YP2

and YP3 respectively are derived from independent primary translation products by removal of signal peptides and addition of carbohydrate, phosphate and sulfate (Brennan et al, 1982; Minoo and Postlethwait, 1985; Dimario et al., 1987), but in contrast to most other insect Vgs, are not processed from large precursors. Yp patterns of this type have been found in several other higher Diptera, including blow fly *Calliphora* (Jensen et al, 1981) flesh fly *Sarcophaga* (Huybrechts and DeLoof, 1982), med flies *Ceratitis* (Rina and Mintzas, 1987) and melon fly *Anastrepha* (Handler and Shirk, 1988).

In addition to the Vgs and YPs, other proteins may be present in varying proportions in insect yolk. Some are female specific and taken up selectively from the hemolymph, suggesting that they may have specific roles in the egg. Eggs of *H. cecropia* contain large amounts of vitellin, and lesser amounts of lipophorin (a non sex-limited lipid transport protein) and a small 30 kD protein called microvitellin (Telfer and Kulakosky,1984; Telfer and Pan, 1988). In *M. sexta* similar size classes of proteins are found, together with a blue biliprotein, insecticyanin (Law,1989). In *M.sexta* mVg (31 kDa) production by fat body is limited to the female (Kawooya and Law, 1983), beginning in the late larval stages (Law, 1989). The silkworm, *B. mori*, is unusual in that a group of proteins of about 30 kD that appear first in the last larval instar (Izumi et al, 1981), remain at high quantities in the pupal stage and are taken up into the oocytes to become abundant in yolk as the vitellin (Mori et al, 1989; Zhu et al, 1986).

In *L. migratoria*, the hemolymph contains low levels of a 21 kD monomeric protein which like Vg, is produced only in adult female fat body and taken into the developing oocytes (Zhang and Wyatt, 1989). In *Chironomus* spp., hemoglobins in general, and female specific hemoglobins contributing to the yolk (Trewitt et al, 1986; Fabb et al, 1990) have been identified.

B. Follicle specific proteins:-

In addition to their permissive role in vitellogenesis, the ovarian follicle cells of some dipteran and certain lepidopteran species were shown to synthesize significant

amounts of yolk proteins during oogenesis which contribute to the protein stores of the mature egg along with the extra ovarian vitellogenins (Bast and Telfer, 1976; Telfer and Anderson, 1969; Brennan and Mahowald, 1982; Borovsky and Van Handle, 1980). Identification of paravitellin as a follicle cell product in *H. cecropia* by Telfer et al.(1981) was the first report of the role of the ovarian follicle cells in the production of yolk protein. Later, Brennan et al (1982) showed by *in situ* hybridization, that in *Drosophila* as well, the yolk protein genes are expressed in the ovarian follicle cells. These were the same three yolk polypeptide genes, yp1, yp2 and yp3 expressed in the fat body cells. Subsequently, in the stable fly, *Stomoxys*, all the yolk proteins (vgs) were shown to be made by the follicle cells (Chen et al, 1987). However in lepidopterans different sets of yolk protein genes are expressed in the fat body and ovarian follicle cells.

Yolk proteins synthesized within the ovaries vary in size between different lepidopteran species and have been given different names, e.g. paravitellogenin (70 kDa) in *H. cecropia* (Telfer *et al.*, 1981); egg-specific protein [ESP (72 kDa)] in *B. mori* (Irie *et al.*, 1983); follicular epithelial yolk polypeptides (FEYP) in the pyralid moths (Zimoskowa et al,1993). In the pyralids *Galleria mellonella* and *Plodia intepunctella* the, proteinaceous yolk consists of four yolk polypeptides (YPs) designated as YP1, YP2, YP3 and YP4 in descending order of molecular weight (Shirk *et al.*, 1987; Bean *et al.*, 1988). In *Galleria*, the molecular weights of these four yolk polypeptides as determined in SDS / PAGE are 159, 74, 44 and 37 kDa. Shirk (1987) has shown by *in vivo* and *in vitro* ^{35}S-methionine labeling studies that in the pyralid moths, YP1 and YP3 are synthesized in the fat body cells, where as YP2 and YP4 are synthesized by the ovarian follicle cells. Although eggs of *H. cecropia* contain four major yolk proteins, only vitellin, derived from the fat body appears to have a composition similar to that of YP1 and YP3 of the pyralid moths. Paravitellin (70 kDa), produced in the follicle cells in *H. cecropia*, is similar in size to YP2 and exists as a 70 kDa monomer in the yolk. In *B. mori,* egg specific protein (ESP) a 72 kDa polypeptide was shown to be produced by the ovarian follicles during vitellogenic

stages (Sato and Yamashita,1989). However, except for the similar size of proteins and site of expression, the relationship of these yolk proteins is not known.

Yolk proteins synthesized within the ovaries seem to differ also in their subunit composition. While paravitellin of *H. cecropia* do not form any intermolecular associations, the ESP of *B. mori* was found to form a trimer with its 55 kDa proteolytic product (Yamashita and Indrasith, 1986). According to Bean et al (1988) and Zimowska et al, (1993) the molecular mass of native protein consisted of YP2 and YP4 in *Plodia interpunctella* . Based on gel permeation chromatography (Shirk et al,1984) and pore limiting electrophoresis (Bean et al, 1988) the molecular weight of the native proteins are 264 kDa or 237-235 kDa respectively. A smaller species of YP2 / YP4 hetero dimer with molecular mass of 93 kDa - 98 kDa was also found suggesting a 1:1 ratio of YP2 / YP4, and the larger 264 kDa species are probably multiple of this dimer. The variability in larger YP2 / YP4 species (235 to 264 kDa) is believed to be due to either the proteolytic cleavage of YP2 or removal of weak subunit associations. By immunoflourescent labeling of whole fixed ovarioles and immunogold labeling of ultra- thin section of egg follicles, Zimowska et al (1991) and Shirk et al (1987) have shown the accumulation of follicle specific yolk polypeptides in the yolk spheres of *Plodia.*

Microvitellin, produced in the fat body of both sexes is similar in size to YP4, which exists as a monomer in the yolk (Telfer, 1981) of *H. cecropia.* Neither paravitellin nor microvitellin (mVg) appears to form any higher subunit associations. In *M.sexta.* also microvitellin does not form any higher subunit associations. In *M. sexta*, unlike in *H. cecropia*, mVg is only synthesized in the female fat body (Kawooya et al, 1986). A smaller polypeptide similar to YP4 of the pyralids has not been described in *B. mori* or *H. cecropia.* Recently, follicle-specific proteins (FSPs) FSP-1 and FSP-2 which are synthesized at the end of vitellogenesis have been identified in *M. sexta* (Tsuchida *et al.*, 1992). FSP-1 is a dimer of a 65 kDa subunit polypeptide and FSP-2 is a heterodimer composed of 140 kDa and 35 kDa polypeptides. Based on the native and denaturing gel

electrophoresis, the fat body specific yolk proteins and the follicle specific yolk proteins do not seem to form subunit associations with each other in any of these insects.

The yolk proteins produced by the follicle cells are also believed to follow the same route to accumulation in the same yolk platelets (Zimowska et al, 1993) as the fat body specific vitellogenins. Using monospecific antisera for each of the yolk protein subunits Bean et al (1988) and Shirk et al (1992) found that, in *P. interpunctella* a, a moth closely related to *G. mellonella*, the follicle specific yolk protein YP2 was the first to accumulate in the ovaries by day 4 after pupation. Secretion of YP2 by follicle cells towards the oocyte brush border allows YP2 to be taken up by the oocyte as soon as synthesis begins and does not require the development of patency by the follicular epithelium. Even though the synthesis, YP1 and YP3 in the fat body, already can be detected by immunoflourescence (along with YP4 synthesis) in follicle cells these proteins do not start accumulating in the yolk sphere until 20 hours after the initiation of YP2 accumulation (Zimowska et al, 1993). Based on these studies, an extended period termed the provitellogenic stage has been determined for moth oogenesis. Thus moth oogenic stages may be summerized as follows: 1) in the previtellogenic stage, the follicular epithelium is not patent, and the oocyte lacks yolk spheres, 2) in the provitellogenic stage, the follicular epithelium is still not patent, but yolk spheres with limited yolk proteins begin to appear, 3) in the vitellogenic stage, the follicular epithelium is patent, and the yolk spheres contain all the yolk proteins. The initiation of active yolk uptake and the formation of yolk spheres by the oocyte prior to the initiation of patency by the follicular epithelium was suggestive of a stepwise mechanism of activation of the vitellogenic processes. Zimowska et al (1993) also found a concurrent increase in YP4 protein levels with the development of patency suggesting that a common factor(s) / mechanism (s) of activation initiates both these events, i.e; YP4 gene expression and development of patency. At the end of vitellogenesis, patency is terminated by the formation of an occlusion zone between the follicle cells (Rubenstein, 1979). A second route of molecular transport between follicle cells and the vitellogenic oocyte may also

exist. High permeability junction complexes between the follicle cells and oocytes have been discovered (Woodruff, 1979), and are believed to allow passage of small hydrophilic compounds directly into the oocyte cytoplasm. However, little information is available regarding the factors involved in the formation of these complexes.

At the late vitellogenic and choriogenic stages of follicle development (Pefereon and DeLoof, 1986; Kafatos et al, 1987a and 1987b), the follicle cells express egg shell membrane (Popodi et al, 1988) and chorion proteins (Kafatos et al, 1987a and 1987b). Thus, ovarian follicle cells, provide an ideal system to study regulation of sequential gene expression and to analyze the molecular mechanisms of gene expression in a differentiated tissue.

Regulation of oogenesis and vitellogenesis

The factors that regulate synthesis of yolk proteins (YPs) vary in different groups of insects. Juvenile hormone (JH) and ecdysone (20-HE), the two major hormones that control the metamorphic developmental changes in insects, were shown to regulate yolk protein synthesis in some insects. In cockroaches (Koeppe, 1976), locusts (Locke et al,1987) and a variety of coleopterans and hemipterans (see, Koeppe, 1985), YP production is controlled by juvenile hormone. Ecdysone was, however, found to control YP synthesis in the dipteran mosquito *Aedes aegypti* and *Sarcophaga bullata* (Cardeon et al,1988; Huybrechts and DeLoof,1981). In *Drosophila* (Postlethwait and Jowett,1981), ecdysone was shown to control YP gene expression, but this regulation is dependent on the state of the sex determination genes (see; Postlethwait and Kunert,1986). Follicle specific expression was shown to be stimulated by JH. In most lepidopterans, no hormonal or known developmental cue for the regulation of YP gene expression has been identified (see, Koeppe, 1985; Wyatt, 1991).

In *B. mori* , an increase in ecdysteroids early in the pupal stage is necessary to initiate adult development (Hanoaka and Ohnishi, 1974). In addition, treatment of isolated

pupal abdomens with ecdysteroids showed that initiation of follicular differentiation and ovarian growth is dependent on the early pupal ecdysteroid peak in the silkworm, *B. mori* (Ohnishi, 1987; Tsuchida et al, 1987). Late in pharate adult development, a decline in ecdysteroid titer is required for completion of the metamorphic process (Hanoaka and Ohnishi, 1974).

In light of information concerning ecdysteroid secretion by ovaries (Hanaoka and Ohnishi, 1974; Hagedorn et al, 1975; Bollenbacher et al, 1978) and the need for ecdysone in ovariole development, the question of endocrine control in ovariole maturation and oogenesis has been reopened amid some controversy. *In vitro* incubation in 20-HE (20-Hydroxy ecdysone) was shown to induce egg follicle development and differentiation in the last instar larval ovaries in *G. mellonella* (Shibuya and Yagi, 1972). However, the ecdysone level goes down in *G. mellonella* by the fourth day after the larval- pupal molt, followed by a female specific rise due to an ovarian production of ecdysteroids which was observed in the late pharate adult (Plantevin, 1984). Recently studies in *P. interpunctella* have shown that the ovaries require the presence of 20-HE to support normal levels of YP2 synthesis, but that a concentration higher than the physiological level of 20-HE suppresses the accumulation of translatable YP2 mRNA and yolk protein synthesis in the ovaries (Shirk,1987).

There are two alternatives that could explain the onset and implementation of the developmental program leading to oogenesis and vitellogenesis in moths. Either the ovarian developmental program could be autonomously implemented at a specific developmental stage by coupling it to the onset of pupation or initiation of pharate adult development, or oogenesis may be initiated by an extra-ovarian female specific humoral factor produced in some other tissue (brain, gut etc.) in response to a signal from ovary.

The presence of residual levels of Vgs in the males of some lepidopterans including *Galleria* was reported (Telfer, 1954; Lamy, 1986; Irie and Yamashita, 1983). It was suggested that transplanted ovaries were able to develop and undergo vitellogenesis in the

male hosts of *Galleria* because males also produce Vgs (see, Lamy, 1986). This could mean that the ovary is able to induce the male fat body to increase expression of the Vgs. On the other hand, in the gypsy moth *Lymantria dispar,* while ovaries transplanted into males developed and oogenesis appeared normal, the oocytes were found to accumulate male fat body proteins rather than vitellogenins (Ballarino et al, 1991). In light of the information regarding synthesis of yolk polypeptides by the ovarian follicles it would be interesting to see whether vitellogenesis in ovaries transplanted into males is dependent on Vgs produced by the male fat body or solely on the accumulation of follicle specific yolk polypeptides. Normal follicle-specific gene expression in the male milieu would suggest that either no female specific humoral factor is involved, or that these factors are produced by the ovarian tissue. The other possibilities could be that either the oogenic developmental program is autonomous, or the ovaries need a non-sex specific developmental signal which allows it to implement the oogenic developmental program autonomously. The availability of a cDNA encoding the follicle specific YP2 and/ or YP4 yolk protein (s) will help to answer some of these questions.

Nucleotide and Amino acid sequence analysis of insect egg proteins

Vitellogenic egg protein genes or cDNAs of a limited number of insects have been isolated thus far: *D. melanogaster* YPs (Bownes,1986; Garabedian et al, 1987); *Ceratitis capitata* (Rina and Savakis, 1991), *M. sexta* microvitellogenin (mVg; Wang et al,1989), 30 kDa proteins (Mori et al,1989) ESP (Inagaki and Yamashita, 1989) of *B. mori*, *Anthonomus grandis* Vg (Trewitt et al,1992) and *A. aegypti* vitellogenic carboxy peptidase (VCP; Hays and Raikhel,1990). Partial sequences of locust (Lock et al, 1987) and mosquito (Gemmill et al,1986) vitellogenin genes are also known. Among these only the *Drosophila* YPs and *B. mori* ESP are expressed in the ovarian follicle cells. The available data suggest that insect yolk proteins may have diverse evolutionary history. For example, the *A. aegypti* VCP amino acid sequence derived from the cDNA shows a high

degree of similarity to several serine carboxy peptidases of mammals (See; Raikhel and Dhadialla,1992). The nucleic acid sequences of *M. sexta* mVg are 70% similar to the sequence for the 30 kDa serum proteins of *B. mori* (Sakai. et al, 1988). The female specific mVg from *Manduca sexta* was found to be related to non-sex-specific *B. mori* 30 KDa hemolymph proteins (Wang et al,1989). It is suggested that the *B. mori* 30 kDa protein genes were primitively yolk proteins, and in the amplification process, a prototype gene lost its female specific elements (Mori et al, 1989). In support of this notion, transposon-like Bm1 repetitive elements were found in the vicinity of tandemly arranged *B. mori* 30 kDa protein genes (Mori et al, 1989).

The *A. grandis* Vg gene structure, particularly the position of cysteine residues, necessary for structural integrity, were found to be conserved when compared to nematode and vertebrate Vg genes (Trewitt et al,1992). On the other hand, the *D.melanogaster* YPs show homology to triacyl glycerol lipases of mammals (Tepstra and Greet, 1988). Thus, it has been suggested that the higher dipterans are different from other insects in that they have lost the ancestral Vg genes and have recruited an entirely different gene to serve this purpose. Among follicle-specific yolk proteins, only the cDNA encoding the ESP of *B. mori* has been isolated and sequenced. Sequence comparisons of ESP revealed no similarity to any other known yolk protein sequence. The isolation of a cDNA and obtaining the nucleotide sequence of follicle specific yolk proteins in *Galleria* a pyralid, which is evolutionarily separated from the lineage that gave rise to saturnids (*B. mori, H. cecropia, A. cynthia* and *M. sexta*) can provide information regarding the evolutionary history of these yolk proteins.

Recent studies also suggest that yolk proteins might serve other functions such as carriers of conjugated ecdysteroids or enzymes (Bownes et al, 1989; Inagaki and Yamashita, 1989, Hagedorn, 1989). The study of yolk protein genes may also help to elucidate the significance of the differences in tissue specificity of yolk protein gene expression in the moths.

MATERIALS AND METHODS

Insects:

The culture of *Galleria mellonella* L. (Lepidoptera: Pyralidae) was maintained at 29°C under a 14 / 10 hour dark and light cycles, 60 to 70% relative humidity (Sehnal, 1966; KrishnaKumaran, 1972). They were raised on an artificial diet containing honey, beeswax, Gerber's mixed baby cereal, yeast and glycerol. Eggs were collected regularly and placed in 150 mm petri dishes with food. Once the larvae hatched and developed into 4th instar grown to about 10-20 mg size, they were transferred to petri dishes containing fresh food and maintained at low density (300- 400/ 150 mm dish). The last instar larvae were collected as soon after their molt from penultimate larvae. The pupae were collected soon after ecdysis. Both last instar larvae and pupae were staged from the time of collection and the day of molt is designated as day zero (D0) and 24 hrs. later the insect is designated as one day old (D1). The last instar larvae are maintained at lower density (12-15 / 90 mm dish) since growth and development is affected by crowding. Day seven last instar larvae, are placed in a large plastic container on a corrugated cardboard for pupation when they spin a cocoon and pupate inside the cocoon. The pupae initiate adult development within 36 hrs. after pupation and the adult development is completed in 11 days at room temperature 22 - 24 ^{0}C and 8 days at 29 ^{0}C depending on the and sex.

Tissues:

Although adult development in *Galleria* is uniform, in order to insure selection of ovarioles of the correct developmental stage, the appearance of number of morphological features, visible under a microscope was correlated with the development of the ovary (Table.1). These features were carefully examined for selecting the animal for studying and for dissection. The ovarioles were examined before using the tissue for analysis to confirm their normal development. For RNA isolation, day-8 pharate adult ovarioles were

ground to fine powder in liquid nitrogen. When egg follicles of specific stage were needed, ovarioles from pharate adults were dissected to provide the specific stages of oogenesis based on the size of the egg follicles (Table.2). Tissues were dissected in ice cold Ringer's solution or Grace's fresh culture medium (BRL) from males or female pharate adults and larvae of specific age as described in the text. The insects were surface sterilized with 80% ethanol immediately before use or storage in liquid nitrogen.

Construction of cDNA Library

i) Poly A+ RNA Isolation:-

Poly A + RNA was isolated from day 6 to 8 pharate adult ovarioles (vitellogenic stages, see Table.1) pulverized in liquid nitrogen, using the Quick prep mRNA Kit (Pharmacia PLBiochemicals) following the manufacturer's protocol. In brief, this procedure involved deproteinization of liquid nitrogen pulverized tissue, by guanidium thiocyanate in the N-lauryl sarcosinate homogenization buffer. After centrifugation at 10,000 rpm for two minutes, to remove the debris, the supernatant was diluted with an equal volume of the elution buffer (Tris-HCl,) and oligo-dT cellulose (25mg/1 ml) was added. After 10 to 15 minutes incubation, the poly A+ RNA bound oligo-dT was washed several times with high salt buffer (10mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl) and centrifuged for 5 seconds each time. Finally, the oligo-dT with the bound poly (A)+ RNA was transferred to a spun column. RNA isolated from approximately, 100 mg of insects or tissues was processed in each disposable column. After three washes with low salt buffer (10mMTris-HCl, pH 7.5, 1mM EDTA, 0.1 M NaCl). Poly(A)+ RNA was eluted with elution buffer [10 mM Tris-HCl (pH 7.5), 1mM EDTA] at 65 ^{0}C. The eluate was made 0.25M with potassium acetate (pH 5.0) by the addition of 1/10 volume of 2.5 M

TABLE I: Developmental changes in the ovary during adult development of *Galleria mellonella.*

Pupal age (Days) at 24°C Male	Female	Anatomical structures & morphological features	Developmental-changes in the ovary.
	D0	White pupae (soft cuticle)	Ovary appears as a sphere form- referred to as gonadal cysts.
	D2	Tan pupae (cuticle sclerotized) clear eyes (eyes not pigmented)	
	D3	Tan pupae dark brown eyes (pigment spots appear in the eyes). Fat body dissociates	Ovarioles start to exfoliate. The four tubular structures protrude through the ovarian sheath. The germarium is visible at the tip of each ovariole.
	D4	Tan pupae with black eyes and yellow tarsal claws	The ovarioles elongate. Follicles differentiate inside the ovarioles. Nurse cell and oocytes are distinguish-able in the terminal follicles.
	D5	Tarsal claws brown (Black in males) dissociated fat body Tracuoles visible.	Translucent yolk deposition in the terminal follicles.
	D6	Brown prolegs Tarsal claws black	Milky opaque yolk deposit appear in the terminal follicles.

D8	Wings brown (black in males) fat body reassociates	2/3 of the follicles in each ovariole has yolk deposited (Active vitellogenesis).
D9/D10		
	Dark brown / black pupae with dark brown wings and black scales	Vitellin membrane formation in terminal follicles.
	Newly eclosed adult (female)	4/5 ovarian follicles contain yolk. Terminal 10 to 12 follicles (in calyx) have chorion deposited.

* Adult males eclose one day earlier than females.

Table 2: Stages and dimensions of egg follicles in *Galleria mellonella*

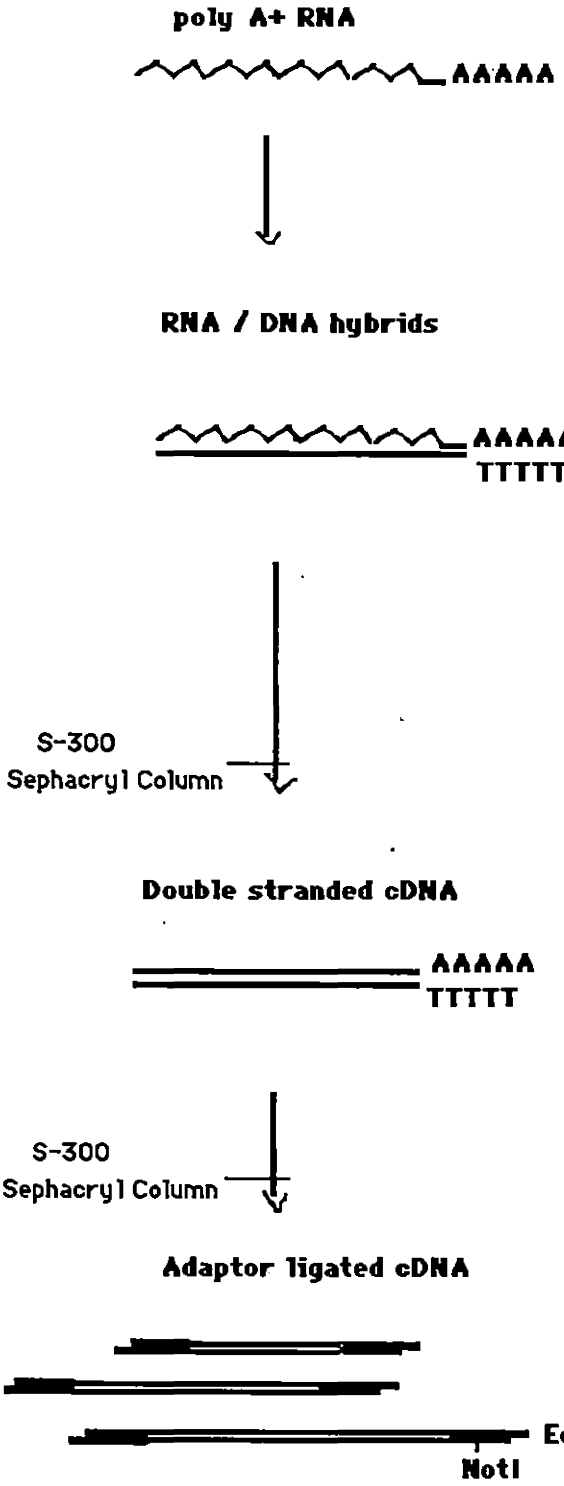
Oogenic Stages	Number of egg follicles in D8 pharate adult ovarioles	Terminal Follicle Size
Pre-vitellogenic: (S1-3)		
Nurse cells & oocyte differentiated by S3. Posterior localization of ocyte, surrounded by follicle cells.	60 - 80	0.02 - 0.04 mm (0.02mm, oocyte)
Vitellogenic:(S5-S6)		
Oocyte accumulate translucent yolk (1/2 filled) Prominent Nurse cells.	80 - 100	0.08 -- 0.15 mm (0.04mm, oocyte)
Late vitellogenic:(S7-S10)		
Opaque, yellowish yolk accumulation. Oocyte enlarge. Nurse cells appear as caps on top of the follicle.	60 - 100	0.2 -- 0. 30 mm (0.18 - 0.26mm,oocyte)
Choriogenic: (S11-S12)		
Nurse cells complete discharging their contents into oocytes. Compressed (flattened) and disappear. Vitellin membrane formed and chorion membrane formation started in terminal follicle	~ 20 (in newly eclosed adult)	0. 35 -- 0.40 mm (oocyte, 0.3 - 0.4mm)

potassium acetate, and ethanol precipitated. Glycogen (10 mg / ml) was added, 1/40 volume, to promote precipitation. The RNA precipitate was recovered by centrifuging in a microfuge for 20 minutes. After washing with 70%, ethanol RNA was dissolved in DEPC treated H_2O and stored at -70^{0}C.

ii) Double strand cDNA synthesis:-

A *Galleria* ovariole cDNA library was made starting with 2µg of Poly A+ RNA isolated from vitellogenic ovarioles. A flow chart showing the main steps for making vector ready cDNA is given in figure-1a. First strand cDNAs were synthesized from oligo-dT primed poly(A)$^+$ RNA by the procedure of Gubler and Hofman (1983), using MMLV reverse transcriptase at 37°C (Pharmacia, LKB). The cDNA was spiked with ^{32}P-dCTP, to monitor synthesis and size analysis of the cDNA product (Fig. 1b). The first strand cDNA synthesis is described in detail under the section- "preparation of labeled single strand cDNA probes" on page- 31 . The second strand was synthesized using DNA polymerase I in the second strand synthesis buffer, and blunt ended by Klenow fragment in the buffer. The average lengths of the first strand and second strand cDNAs were estimated on the basis of their migration in alkaline gel electrophoresis. The product yield was calculated by TCA precipitation of aliquots from each of these reactions. After an extraction with phenol/chloroform the cDNAs were purified by passing through an S-300 Sephacrylspun column.

To the blunt ended cDNAs, EcoR I/Not I adaptors were ligated by incubating at 14^{0}C overnight with T4 DNA ligase, in the ligation buffer (66 mM Tris-HCl, pH 7.6, 1 mM spermidine, 10mM $MgCl_2$, 15mM dithiothreitol, 0.2 mg/ml BSA -DNase free). The adaptor is composed of a phosphorylated blunt end and a nonphosphorylated EcoR I overhang as well as an internal Not I site. Using this adaptor eliminates the need for methylation and EcoR I digestion, normally required at this stage. After phosphorylation



First strand cDNA synthesis

Add

- Oligo-dT primers
- MMLV Reverse transcriptase
- Incubate at 37°C for 1 hr

Second strand synthesis

Add

- RNase H
- E. coli DNA polymerase I
- Incubation at 12°C for 1hr.
- Incubation at 22°C for2 hrs.

Add

- Klenow fragment to blunt end t
- Incubation at 37°C for 30 mi

Adaptor Ligation

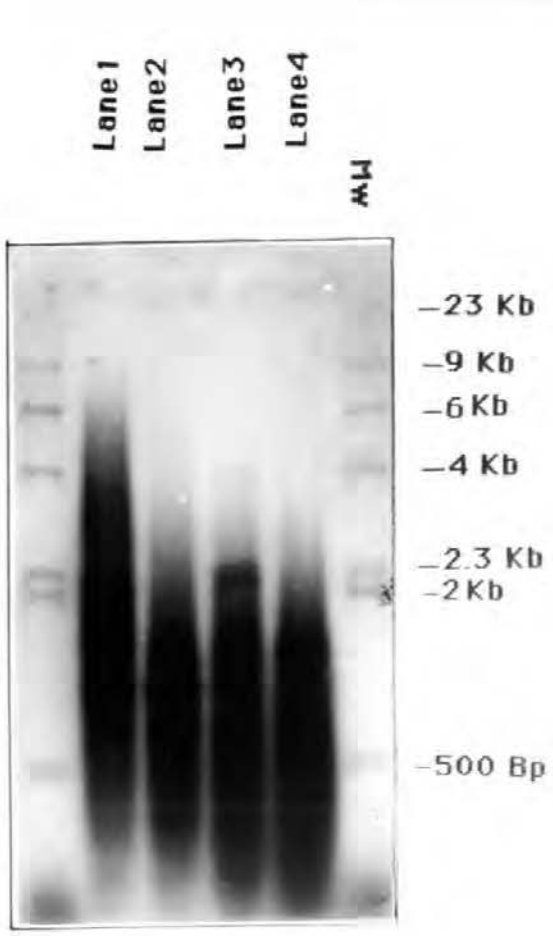
Add

- T4 DNA ligase
- EcoRI-NotI adaptors
- Incubation at 14°C overnig

Main steps of vetor ready cDNA synthesis

FIGURE 1b: Size range and integrity of poly A+ RNA as detected by the first strand cDNA products.

First strand cDNA was generated in the presence of α^{32}P-dCTP using MMLV reverse transcriptase (Pharmarcia, LKB) at 37°C. 1ug of poly A+ RNAs were used as templates and primed with Oligo-dT $_{(12\text{-}18)}$ for the reactions. 1ul sample from each reaction (50ul) was analyzed by electrophoresis on an alkaline 1.2%agarose gel. The gel was blotted dry using 3mm papers and exposed to X-ray film at -70°C for 6 hrs. The lanes show cDNAs derived from 1) vitellogenic ovariole, 2) day 8 pharate adult abdomen (- ovariole), 3) day five last instar larvae and 4) day zero male pupae. The end lanes on both sides of the gel contain the Hind III digested Lambda DNA end labeled with α^{32}P-dATP, as size markers.



of the EcoR I ends with the addition of ATP and T4 polynucleotide kinase and removal of free adaptors and short cDNAs (<300bp), by passage through Sephacryl-300 exclusion spin-column (Pharmacia, LKB) the cDNAs were ready for ligation to lambda gt10 arms (stratagene). The concentration of cDNA yield was measured using an aliquot on an ethidium bromide / agarose plate along with known standards.

ii) Ligation and packaging of the library:-

The cDNAs with EcoR I overhang were cloned into Lambda gt10 vector following the protocols of the supplier (Stratagene). The lambda gt10 vector has a single EcoR I site within the cI repressor gene, leading to inactivation of the lambda repressor upon cloning of an insert. Therefore, the recombinant phage are lytic and produce clear plaques where as the parental phage are lysogenic and produce turbid plaques. The lambda gt10 vector, as supplied by the manufacturer (Stratagene), was EcoR I digested and dephosphorylated arms. The cDNAs (average 1.5 kb) and the lambda gt10 arms (43 kb)were mixed in a ligation reaction in a molar ratio of almost 1: 1. A 10µl ligation reaction contained, 1.0 µg (0.07pmole) of lambda gt10 arms, 0.06µg (0.06 pmole) cDNA and 1 unit of T4 DNA ligase in 40 mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 0.5 mg/ml BSA and was incubated at 4 °C overnight. A control reaction without insert DNA was also carried out.

The recombinant DNA was packaged in vitro into infective lambda particles using Gigapak- Gold packaging extracts from Stratagene, according to the manufacturer's protocol. In brief, 3.3 µl of ligation reaction mixture containing about 0.33 µg of recombinant DNA was used for each packaging reaction. It was first added to a freeze/thaw extract which was then mixed to the sonic extract. The mixture was incubated at 22 °C for 2 hrs., and the reaction was terminated with the addition of 0.5 ml of SM medium [50mM Tris-HCl (7.5), 100mM NaCl, 10mM MgSO4. 0.01%gelatin)]. Two

such packaging reactions were pooled to make up a library with a total volume of 1ml. After addition of 30µl chloroform, the library was titered according to standard procedures using C600 Hfl and LE 392 (OD600 = 0.5) cells as lawn.

The E.coli bacterial strain, C600 Hfl (tetracycline R) is a restrictive host and allows only the recombinant plaques to form. E.coli strains LE392 or C600 are permissive hosts and allow both parental and the recombinant phages to form plaques. The high frequency of lysogeny mutation in C600hfl represses c1+ (parental) phage 50 to 100 fold. Bacterial strain E.coli C600 Hfl was used for titering the library and LE392 was used for rescreening and plaque purification procedures. The library was found to contain 6.0 x 10^5 pfu / ml.

Screening For Ovary Specific cDNA clones

i) Plating recombinant phages and membrane lifting of phage DNA:

An overnight culture of E.coli C600 Hfl and LE392 were grown at 37 °C in TB (0.5% NaCl, 1% tryptone) or LB media containing 10mM MgSO4 and 0.2% maltose, and allowed to grow to mid- to-late log phase (OD600 ~ 1.0). The cells were pelleted by centrifugation at 1,500 x g for 10 min and re suspended in 10 mM MgSO4 for a final OD600 = 0.5. Five microliter aliquots of the library (~3000 PFU) were adsorbed to 60µl of (OD600=0.5) E.coli C 600 Hfl or LE 392 cells for 20 minutes at 37°C, mixed at 47°C with NZY (1% type A casein hydrolysate, 0.5 % Nacl, 0.5% yeast extract, 8 mM $MgSO_4$ and 0.7% agarose) soft agarose, and poured on 85 mm LB agar petri plates. After incubation at 37°C for 8 to 12 hrs, until plaques were 0.5 to 1.0 mm in diameter, the plates were cooled to 4°C.

A nitrocellulose (NC) filter was carefully placed over the lawn until the filter was uniformly wet, and left in place for 2 to 3 mins. The filter position on the plate was marked

by pricking a needle through the filter and the agar at three positions. From each plate two NC filters were lifted for differential hybridization (see below) with ^{32}P labeled positive and negative cDNA probes (see below for preparation). The lifted filters were placed with phage DNA side up, in 0.5 M NaOH, 1.5 M NaCl for 2 to 3 mins to denature the DNA. They were then neutralized by immersing in 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl for 5 mins. The filters were rinsed in 2 x SSC (1x =0.15 M NaCl, 15mM Na citrate, pH 7.0), air dried, baked in vacuum at 80°C for 1 to 2 hrs, and stored desiccated at room temperature.

ii) Single Strand cDNA probes:

Single strand $^{32}P\alpha$-dCTP labeled cDNAs were made starting with Poly A+ RNA (5- 10µg/ 3ul DEPC H_2O) isolated from the specific sex, stage or tissue. The RNA was heat denatured at 70°C for 5 minutes, chilled on ice, followed addition of 2µl oligo $(dT)_{12-18}$ (1mg/ml) was added and allowed to anneal. Then the following were added at 4°C in a 25µl reaction: 4µl DEPC H2O, 1µl RNase inhibitor (Promega, Madison), 2.5 µl 10x reverse transcription buffer (50mM Tris-HCl, pH 7.6, 10 mM MgCl2), 0.5µl 1M KCl, 1µl dNTPs (-dCTP) 20mM each, 2.5 µl 0.1M dithiothreitol, 5ul of [α-^{32}P]dCTP (3000Ci / mmol). The reaction was stopped by the addition of 1µl each of 0.5M EDTA and 10% SDS. The RNA strand was degraded by the addition of 1.5µl of 3N NaOH and incubation at 68 °C for 30 mins. Following neutralization with 5µl Tris-HCl (pH 7.4), the unincorporated nucleotides were removed by passage through a sephadex G-50 column.

iii) Differential screening:-

Since ovariole specific messages are expected to be present only in the ovariole but not in the male, ovariole specific clones were isolated by differential plaque hybridization,

using ovariole and male pharate adult poly A+ RNA were used to generate first strand cDNA probes following the protocol of St. John & Davis (1979). Duplicate nitrocellulose filter lifts were probed with ^{32}P labeled first strand cDNA probes generated starting with Poly A+ RNA isolated from either ovariole [(+)ve] or male pupae [(-)ve]. After, hybridization to the (+)ve and (-)ve probes and exposure to X-ray films, the positive signals on the films were matched by alignment to the corresponding areas on the plates. The differential signals of plaques that are positive with (+)ve cDNA but not hybridizing to (-)ve probe, were identified by comparing the signals produced by the two filters lifted from a single plate.

For secondary screening, the putative ovariole specific phages from each single plaque that gave a differential signal, were toothpick- transferred onto a gridded 85mm LB agar plate seeded with an *E.coli* LE392 lawn. After infection, lysis and plaque formation, the phages were lifted on to NC filters in duplicate to hybridize to the (+) ve and (-) ve probe cDNAs. Plaques showing signals with only the positive probe and not with the (-) ve probe, even after second screen were isolated by removing the agar plug containing the phages with a pasture pipette, and suspending it in SM medium. Phage isolates from the second screen phages were plated at low density and were screened using ovariole and female minus ovary RNA derived probes. The selected plaques were isolated as agar plugs in 1ml of SM and after the addition of 30µl chloroform, stored at 4°C.

After screening $20x10^3$ plaques, seventy eight putative female specific clones that hybridized to ovariole cDNA probe but not to male derived probe were isolated. After a second round of differential screening, forty five ovariole specific clones were obtained. Another round of screening with single strand cDNAs derived from ovariectomized pharate adults as negative probe, confirmed thirty six clones to be ovariole specific. They were numbered one to thirty six (Ov # 1-- Ov# 36).

iv) Lambda Phage DNA isolation:

Recombinant phage DNA was affinity purified using Lambdasorb (Promega) according to the supplier's procedure except that the SDS step was omitted. For large scale phage DNA isolation, the phage were isolated by CsCl gradient centrifugation and DNA was extracted by the formamide method of Davis (1980); see below.

v) Subcloning:

The lambda gt 10 recombinant clones are equipped with EcoR I sites on both on both sides of the cDNA insert. To purify the cDNA inserts EcoR I was used for digestion, the resulting fragments were fractionated on agarose gels (see page 32 for procedure), and the insert DNA was eluted from agarose gel slices. The cDNA insert from the gel was isolated by the freeze squeeze method or by binding to glass milk (Bio 101). Inserts from the lambda clones were then subcloned into EcoR I site of plasmid pGem-3Z (Promega). Plasmid pGEM-3Z is a 2.7 Kb vector with 1) a multicloning site with EcoR I site at the 5' and Hind111 site at 3' and 2) T7 and SP6 RNA polymerase promoter regions at 5' and 3' ends of the multi cloning site respectively, situated in frame within the Lac-Z coding region and an 3) Ampicillin resistance gene for selection of transformants. The T7 and SP6 RNA polymerase promoters allow transcription from either orientation of the insert and thus sense or antisense RNA probe synthesis. In addition oligomers of the T7 and SP6 promoter regions are commercially available for use as primers during sequencing reactions. Purified insert DNAs having EcoR I overhangs was ligated into EcoR I sites of the pGEM-3Z vector. The inserts were ligated to the EcoR I digested plasmid, typically overnight at 12^0C in a 10µl volume containing vector DNA and insert DNA (at a molar ratio of 4:1 insert vector), 1 unit T4 DNA ligase in 30 mM Tris (pH 7.4), 10mM $MgCl_2$, 10mM dithiothreitol, and 1mM ATP. A volume of ligation reaction containing the

equivalent of 1- 3 ng of vector DNA was used to transform competent *E.coli* , TBI cells. These subclones were transformed into E. coli bacterial strain TB1 or DH5α by the calcium shock procedure (Sambrook et al., 1989). Breifly the cells were grown to an $O.D_{550}$= 0.25 and placed on ice. The cells were pelleted at 40^{0}C for 3 min at 2500 x g, and the pellet was suspended in one-half the original volume of 50 mM $CaCl_2$/ 10 mM Tris-HCl (pH 8.0). After 30 min on ice with occasional swirling, the cells were centrifuged again and the pellet resuspended in 1/20th the original volume. In a typical transformation reaction, 1-5 ng vector DNA from the ligation were added to 150µl of competent cells. After chilling on ice for 30 min, the cells were heat shocked for 2 min at 42^{0}C, returned briefly to ice, and then 200µl LB medium was added. The cells were allowed to grow for 30 to 40 min, at 37^{0}C in a shaker. LB/ Ampicillin (100ug/ml) agar plates were spread with 10 ul of 100mM IPTG (isopropyl-b-D-thiogalactoside), and 75 ul of 2% X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) and allowed to soak. When the transformed cells were ready, they were removed from the shaker, poured on the X-Gal plates and incubated at 37^{0}C overnight. The transformants containing recombinant plasmids, form white colonies and easily detected in the background of blue colonies formed by the transformants containing the vector plasmid.

The presence of subcloned cDNA insert in the plasmid was confirmed by Southern blot (see pg. 34 for procedure) analysis with single strand ovariole cDNA probe or by simply digesting with Not I. Since, Not I site is introduced only with the EcoR I adapters used in cDNA cloning and not found in the plasmid vector, this enzyme specifically released the cDNA inserts (provided there are no Not I sites in the cDNA. The cDNAs cloned in this dual promoter (SP6 and T7) transcription plasmid vector were used for sequencing and transcription of single strand probes or to obtain inserts for nick translated probes.

Preparation of Labeled Probes

i) Nick-Translation:-

Plasmid or cDNA inserts were radiolabeled by nick-translation using the reagents in a BRL kit and protocols supplied by the company. A typical 20µl reaction containing 0.1 to 0.3 µg DNA, 20 mM each of dATP, dGTP, dCTP and dTTP, 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10 µg/ml BSA, 50 µCi [α-^{32}P] dCTP (3000 Ci/mmol), 0.8 units DNA Polymerase I and 4ng/ml DNAse I, was carried out at 14 °C for 1 hr. The reaction was stopped with EDTA (25 mM final concentration), diluted to 100µl with TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and unincorporated label was removed by Sephadex G-50 column chromatography. Typical specific activities in the range of 2×10^7 -2×10^8 cpm/µg DNA were obtained. Probes were used immediately or stored for not more than 2 days at -20 °C. They were denatured at 85 °C for 5 minutes immediately prior to use for hybridization to northern or Southern blots.

ii) Antisense RNA Probes:-

cDNA subclones in pGEM- 3Z were linearized using suitable restriction enzymes. Cloning of inserts into the EcoR I site places the inserts very close to the T7 promoter with all the other restriction sites (eg; Xba I, Hind III) in the multicloning site at the other end. For generating linear template for transcription by T7 polymerase, the recombinant plasmid was cut with either Xba I or Hind III. For transcription with SP6 RNA polymerase the cDNA clones were linearized with restriction enzymes which had sites within the cDNA but not in the multicloning site. Using either T7 or SP6 RNA polymerase, [α-^{32}P]CTP labeled sense or antisense RNA probes were synthesized. A typical 20µl reaction contained the following, added in the same order (at room temperature); 4µl of 5x transcription buffer (200 mm Tris-HCl,pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 2µl 100 mM DTT, 0.6µl of RNasin, ribonuclease inhibitor (40U/ µl), 2.5 mM each of rNTPs contained in a 2ul volume (prepared by mixing 1 vol of H_2O with 1 volume each of the 10

mM ATP, GTP, and UTP stocks supplied), 2.4 µl of 100 uM CTP, 1µl linearized template DNA (0.2- 1.0 mg/ml in DEPC treated water), 50 µCi [α-^{32}P] CTP (3000 Ci/ mmol), 1µl of SP6 or T7 RNA polymerase (at 15-20U/ ul) and DEPC treated H_2O to 20µl. After mixing thoroughly the reaction was incubated at 37 °C for 1 hr. At the end of transcription RQ 1 RNase free DNase was added to a concentration of 1U / µg of template DNA and incubated for 15 mins at 37 °C. The reaction mixture was successively extracted with 1 volume of TE saturated phenol / chloroform (1:1) and 1 volume of chloroform: isoamyl alcohol (24:1) by mixing, vortexing for 1 min, and centrifuging at 12,000 xg for 5 mins. The upper aqueous phase was transferred into another tube and the cRNA sample was precipitated by adding 0.5 volume of 7.5M ammonium acetate and 2.5 volumes of ethanol and placing it at -70 °C for 30 mins. The precipitate was pelleted by spinning at 12,000 xg for 10 mins, at 4°C. After washing in 70% ethanol the precipitate was re suspended in 10-20µl DEPC H_2O and stored at -70 °C until use (usually within two days).

iii) Random Primer Labeling with Digoxigenin -dUTP (For *In Situ* hybridization):-

The random primer labeling method (Feinberg and Vogelstein,1984) allows efficient labeling of small (10ng) and large (up to 3ug) amounts of DNA per reaction. The labeling reaction is fast and results in incorporation of digoxigenin-dUTP in the newly synthesized DNA. This method of hapten incorporation in the DNA yields the highest sensitivity in the detection reaction and is very useful for *in situ* localization of messages in fixed tissues.

Linear DNA is labeled more efficiently than circular and super coiled DNA. Thus the cDNA inserts were used instead of the whole plasmid subclone. In all cases the template DNA should be thoroughly heat denatured to enable the efficient priming along the template strand. Heat denatured DNA (50ng) (10 minutes at 95°C) was chilled quickly on ice and was used in the labeling reaction. In a typical reaction of 20µl, the following are

added in a microfuge tube: 10ng -3µg of freshly denatured DNA, 2µl (10x) hexanucleotide mixture [0.5 M Tris-HCl, 0.1$MgCl_2$, 1 mM DTT, 2 mg/ ml bovine serum albumin, 62.5 A_{260} U/ ml hexanucleotides; pH 7.2], 2µl (10x) digoxigenin- DNA labeling mixture [1 mM dATP, 1 mM dCTP, 1mM dGTP, 0.65mM dTTP, 0.35 mM Digoxigenin-dUTP (pH 7.5) and sterile distilled water to bring the volume to 19µl . Finally, 1µl (2U) Klenow enzyme was added and after a brief spin in the microcentrifuge, incubated at 37°C for at least 1hour.

The reaction was stopped by the addition of 2µl of 0.2M EDTA (pH 8.0). The labeled DNA was precipitated with 2.5µl 4M LiCl and 75 µl prechilled (-20°C) ethanol. After 2 hours at -20°C or 30 mins. at -70°C, the 12000xg pellet was washed with 70% ethanol, vacuum dried and dissolved at 37°C in 50ul TE buffer for 30 minutes. The probe was denatured by boiling for 15 to 20 minutes prior to use. This results in the release of smaller fragments (50 to 200 bp average size) of single strand DNA probes and thus allows for efficient penetration into the tissues. The labeled probe was stored at -20°C. It can be stored for at least one year.

Isolation of high molecular weight genomic DNA

High molecular weight DNA was isolated from liquid nitrogen pulverized pre-penultimate larvae. At this stage they contain less fat and the level of polyploidy is minimal. The larvae were starved for at least four hours to reduce the gut contents before use. The powdered larvae were homogenized in buffer [100 mM Tris-HCl (pH 9.1), 200 mM sucrose, 100 mM NaCl, 50 mM EDTA and 0.5% SDS W / V] and heated at 65°C for 30 mins. The DNA was purified from the homogenate by addition of 8 M KOAc to a final concentration of 1M, incubation for 30 min at 0°C, centrifugation (pellet discarded), extraction of the supernatant twice with phenol/ $CHCl_3$ / isoamyl alcohol (25:24:1) and once with $CHCl_3$ / isoamyl alcohol (24 : 1) and ethanol precipitation. At all steps care was

taken to minimize the fragmentation of the genomic DNA (all mixing of solutions was very gentle and wide bore pipette were used to transfer solutions). The DNA precipitate was dissolved at 4°C (1 -2 days) . Finally, RNase was added and incubated for 15 mins at 37°C . When the isolated DNA was run on 0.5% agarose gel the size of the extracted DNA was found to be above 23 kb.

Endonuclease Digestion and Electrophoresis of DNA

For Southern analysis genomic DNA was digested with restriction enzymes, fractionated by electrophoresis (10 to 20µg DNA digest per lane) and blotted on to Zetabind membrane (Cuno Mfg.). Depending on the ovariole cDNA sequence data, different endonucleases that have recognition sites within the cDNA were used. EcoR I, Pvu II and Pst I, hexanucleotide-recognition site restriction enzymes were used to digest 10-20 µg genomic DNA 1to 6 hr at 37°C (in a 200µl volume) for Southern analysis. The extent of genomic DNA digestion was monitored by running 0.5 µg DNA aliquots on 0.7% agarose-ethidium bromide gels. The digested DNA was extracted with phenol and chloroform (1:1), ethanol-precipitated, and re-dissolved in a small volume of gel loading buffer (0.025% bromphenol blue, 2.5% Ficoll type 400).

The digested DNA was fractionated on a 0.6% agarose gel containing 0.5 µg/ml ethidium bromide in TAE (pH 7.2) buffer (40 mM Tris-HCl, 20 mM sodium acetate, 1 mM EDTA). BRL 1kb DNA ladder and high molecular weight markers were used as size markers. The gels were run at 20- 30 volts overnight (12 hrs.) to allow good separation of the DNA fragments.

Southern transfer of DNA to membrane

After electrophoresis, the gel was photographed under UV. The gel was soaked in

0.5 M NaoH / 1.5 M NaCl for 15 min to depurinate the DNA in preparation for alkaline blot-transfer to a nylon membrane. A typical transfer set up consisted of a large pyrex dish containing 10xSSC (1 x SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) and a glass plate across the top of a dish standing in the solution. A strip of 3MM Whatman filter paper, same width as the glass plate, pre-wet with transfer medium was placed on the glass plate with its ends hanging into the solution and served as a wick. The gel was lifted carefully and placed upside down (to maintain the same orientation of the samples) onto the filter paper. A sheet of saran-wrap was spread over the dish to prevent evaporation of the transfer solution. A window of the size of the gel was cut in the saran wrap exposing only the gel. A Zeta-Bind membrane, quaternary amine derivatized nylon (Cuno Manufacturing Co.), cut to the size of the gel, presoaked successively in H_2O and 6x SSC, was placed over the gel. A clean glass rod was rolled over the gel and the filter as each new layer was placed to expel trapped air bubbles. The zetabind membrane was first covered with two layers of 3MM filter papers. A stack of absorbent paper towels was placed over the filter papers which was topped with a glass plate and a weight. After overnight transfer, the membrane was recovered, rinsed in 2 x SSC and air dried. Position of the wells in the gel and the dye front was marked with ink on the membrane. The membrane was then baked for 1 hr at 80°C under vacuum, and stored at room temperature until used.

Northern Blotting of RNA samples

Poly $(A)^+$ RNA samples (0.3ug to 1.5ug) from different tissues or developmental stages, were denatured in 50% formamide, 2.2M formaldehyde and 10mM phosphate and 0.5 mM EDTA for 5 min at 65°C. After chilling in ice, 1/5 volume of 5x sample buffer (0.5% SDS, 0.025% bromophenol blue, 25% glycerol and 25 mM EDTA) was added and electrophoresed (35-60V, buffer recirculated) in 1.4% agarose gel containing 1.1M formaldehyde and 10mM sodium phosphate (pH 7.4). The running buffer was 10 mM

sodium phosphate (pH 7.4), 0.5 M formaldehyde. After electrophoresis (45V, 16 hrs), the RNA was transferred to Nytran membrane (Schleicher & Schuell) as was described for Southern blots, except that precautions were taken to prevent contamination with RNase and the transfer buffer was 20x SSC. The RNA was crosslinked to membrane by UV irradiation or baking according to the manufacturer's protocol. Large molecular weight RNA ladder (BRL) electrophoresed, blotted and stained in 1% methylene blue served as the RNA size markers.

Hybridization of Phage Lifts, Northern and Southern blots

Prehybridization and hybridization were carried out according to the protocols described in Sambrook et al (1989) in 6 x SSC, 5 x Denhardts reagent and 50mg/ml Salmon sperm DNA, at 65°C for 3hrs and overnight, respectively. Under conditions of standard stringency membranes were washed twice at 65°C for 20 min each in 2 x SSC, 0.1% SDS, twice in 0.2 x SSC, 0.1% SDS for 30 min each. For very high stringency conditions, washes were done at 68°C. After washes, membranes were dried and exposed to Kodak X-AR film with intensifying screen at -70 °C.

In the preliminary studies, pGEM-3Z vector into which the cDNA inserts were cloned, was found not to hybridize to any *Galleria* RNA. Thus, Northern blots were hybridized to labeled subclones. Since, the plasmid vectors were found to hybridize strongly to specific genomic bands, genomic Southern blots were probed with Gene Clean (Bio 101, Inc.) glass milk purified cDNA inserts. All DNA probes were radiolabelled with [α-^{32}P] dCTP or dATP by nick translation (see above).

In Situ hybridization of whole fixed ovarioles

Vitellogenic ovarioles were fixed in 4% paraformaldehyde in PBS (PP) for 2hrs at room temperature on a gentle rotating shaker. They were washed thrice in PBS, (15 min each) and were stored in PBST (PBS and 1% TritonX-100) at 4°C until use. The tissue was treated with 0.2N HCl (20 mins. at 25°C) incubated at 2x SSC for 30 min at 70°C followed by a 5 minute proteinase- K treatment at 37°C. After washing in PBS, the tissue was again fixed in 4% PP for 20 min rinsed in 0.1M TEA buffer (Triethanolamine) and was acetylated by incubating in acetic anhydride/TEA solution (25ml acetic anhyride in 10ml TEA) for 20 min.

Prehybridzation was done at room temperature on a shaker in 0.4ml 5X Denhardt's reagent hybridization buffer lacking probe and dextran sulfate. The Digoxigenin-UTP labeled cDNA probe in hybridization buffer (same as pre hybridization buffer) containing 10% dextran sulfate was heat denatured, for 3min. at 85- 90°C and was added to the hybridization solution, and the tissue was incubated overnight at 37°C. The tissue was washed three times at 45°C for 30 minutes, 1hr and 2-6 hr successively. The buffer contained 50% formamide and 1x salts [1x salts = 0.3M Nacl, M Tris. Hcl (6.8), 0.1M $NaPO_4$, 50mMEDTA]

Hybridization of the probe was detected by anti-digoxigenin antibodies, which in turn was visualized by a peroxidase conjugated secondary antibody reaction. The peroxidase activity converted the substrate diamino benzamidine (DAB) in the presence of glucose oxidase to an insoluble red colored derivative. Thus, the cells with red color indicate a positive signal. Control fixed ovariole tissues, lacking the labeled probe cDNA were processed along side the experimental tissues. The control tissues did not produce the red color precipitate.

Polymerase Chain Reaction (PCR)

PCR amplification of *Galleria* genomic DNA and ovariole cDNA were done using a single block thermocycler (Ericomp). One hundred pmoles each of forward and backward primers was used in a 20ul reaction with 150ng of template, 1.5mM MgCl2 and 2U Taq Polymerase (Promega) through 30 cycles of PCR amplification (94°C/1', 60°C/1', 72°C/3'). A thin layer of mineral oil was placed on top of the reaction mixture to prevent evaporation.

[An 18mer , 5'-GGTGTCGAAGTATCTGGC-3' (+6 to +23 bp of cDNA), as forward primer and 5'-GTACATTGTTGTTCTCAGC-3' (+938 to +920 bp of cDNA) as the reverse primer(fig.4) were used in polymerase chain reactions, with both genomic DNA and cDNA as templates - see the results section for details].

DNA Sequencing

Sequencing was performed by the dideoxy nucleotide chain termination method of Sanger (1977) using [α-^{32}P]dCTP and sequenase enzyme Version 2.0 from US Biochemicals (Cleveland, OH) according to the manufacturer's instructions. Sequenase version 2.0 is a genetic variant of bacteriophage T7 DNA polymerase created by in vitro genetic manipulation. The mutant enzyme is defective in the 3'-5' exonuclease activity of native wild-type T7 DNA polymerase, and therefore has a higher processivity and increased stability relative to the native enzyme. The sequencing reaction steps include: 1) initiation of synthesis at one site where a primer oligonucleotide anneals to the template, 2) synthesis in the presence of one radiolabelled deoxynucleotide ([α-^{32}P]-dATP or dCTP, and 3) controlled arrest of synthesis by addition of proper amount of nucleotide specific chain-terminating dideoxynucleotide analogues.

Template grade DNA for sequencing was prepared by purification of plasmid DNA (Sambrook et al., 1989) through Sephacryl- 400 spin column (Pharmacia). The plasmid DNA was denatured for 5 min, in 0.2N NaOH, and then quickly neutralized and ethanol precipitated prior to annealing the universal T7 or SP6 primers. In addition to the preparation of subclones suitable to extend sequence information, sequence specific oligonucleotide primers (purchased from Bio-synthesis Inc) were also used to prime within the sequenced region of the cDNA. Since the sequencing gel that I was using had a capacity to resolve ~300 bp to 400 bp on average, to facilitate sequencing of internal regions beyond the resolution of the sequencing gel, subclones were generated using suitable restriction fragments from the cDNA and sequenced in both directions using the universal T7 and SP6 primers at least twice.

The products of sequencing reactions were electrophoresed on 6% acrylamide-8M urea gels (Sambrook et al. 1989) using an IBI sequencing apparatus. Electrophoresis was carried out for 20,000 - 50,000 V. hr depending on the extent of sequence information required. Usually, the first sample load was allowed to run till the xylene cyanol dye reaches the bottom of the gel before the second sample was loaded. In some case the second sample was loaded after bromophenol blue migrated near the bottom of the gel (depending on the distance of the insert from the primer end). After electrophoresis, the gel was lifted using an old, washed X-ray film to form a firm base and was wrapped in saran wrap, and was exposed for 12-24 hrs at -70°C to Kodak X-ray film. The nucleotide sequence was read off the autoradiogram.

Sequence Analysis

Sequences were analyzed with the Genetics Computer Group (GCG) software package on the Vax computer (Devereux et al, 1984).

Protein Isolation and SDS Polyacrylamide gel Electrophoresis

Fat bodies, ovarioles and eggs were homogenized in phosphate buffered saline (pH 7.2-7.4) or 50mM Tris-HCl (pH 7.4) buffer containing 1mM PMSF and 5 mM EDTA . They were then centrifuged for 10 to 20 minutes at 10,000xg at 4°C and the infranatant layer between the floating lipid layer and the pellet was recovered. Protein concentration was determined spectrophotometrically by the method of Bradford (1976) using BSA as the standard. Aliquots of protein extracts were separated by 8.75% or 5- 20% gradient SDS-poly acrylamide gel electrophoresis. The samples were denatured by boiling with an equal volume of 50mM Tris-HCl buffer at pH 6.8 containing 5% β mercaptoethanol and SDS for 3 minutes in a waterbath. A 5- 20 % SDS-polyacrylamide gradient gel slab (Laemmli, 1970) was poured using a gradient maker (double cylinder). The samples were electrophoresed overnight at room temperature with 5 mA , 80 V. The molecular weight of specific protein bands in the sample were estimated by comparison to the migration of high molecular weight protein markers (Sigma biochemicals) that were run along with the samples in the gel. The yolk polypeptide bands were detected by Coomassie staining (0.1% in 50% methanol) for 5 min, destained in 50% methanol in 10% acetic acid for 5-10 min, rinsed in deionized water.

Electroblotting and N-terminal amino acid sequence determination

To isolate the specific yolk protein band, homogenates of freshly laid eggs were electrophoresed as described above. One lane was cut out and stained with Coomassie blue. The 37 kDa yolk polypeptide-YP4 band was excised and eluted in PBS. The gel purified YP4 polypeptide was again run on SDS-PAGE and electroblotted onto a immobilon -PVDF membrane (Schleicher and Schuell, Keene, NH) following the procedure described by Matsudaira (1987) for N-terminal micro-sequencing. Briefly, the

SDS-PAGE gels were soaked for 5 min in CAPS transfer buffer [10 mM 3-(cyclohexylamino)-1-propane-sulfonic acid with 10% methanol, pH 11.0] and were then subjected to electroblotting to -PVDF membrane for 10-30 min at 0.5A. The PVDF membranes with attached protein were washed in deionized water for 5 min, air dried and stored at -20°C. One lane was stained with Coomassie Blue as described above. The protein band on the other lanes was located by quick wetting of the membrane in 100% methanol and the membrane piece containing the protein was cut out. Sequencing was performed on Applied Biosystems 477A protein sequencing apparatus at the Protein/Nucleic Acid Shared Facility, Medical College of Wisconsin, Milwaukee, WI.

Raising Anti- YP4 polyclonal antisera

Polyclonal antibodies were generated in New Zealand white rabbits by subcutaneous injection of 100- 150 µg of 37 kDa yolk poly peptide (YP4) gel band, (along with acrylamide) in PBS mixed (1:1) with Freund's complete adjuvant (Difco). Following the first injection rabbits were injected at 2 to 4 week intervals with 100 µg of YP4 antigen emulsified with Freund's incomplete adjuvant (1:1). The rabbits were bled through the ear vein, 8- 10 days after the 2nd boost. Following clotting overnight at 4 °C, clots were separated from the crude antiserum by low speed centrifugation. The antiserum was tested by Ouchterlony double immunodiffusion, divided into 500 µl aliquots and stored at -20 °C.

Ouchterlony double immunodiffusion

Wells were cut in 1% agarose in Tris-HCl (7.5), 150 mM Nacl, 2mM EDTA or PBS (pH 7.2- 7.4). Antiseum was loaded in the center well. Outside wells were filled with antigen . In preliminary studies it was established that at least 20 µl of YP4 antisera is needed in the center well to obtain sharp precipitin lines. The set up was placed in a humid

box at 4°C until precipitin line(s) were formed. The gel was washed in PBS and stained with Coomassie blue. Excess stain was washed in 7% acetic acid.

In vivo ^{35}S-methionine labeling of proteins

Females were injected with 10μCi of ^{35}S-methionine (NEN, Boston, MA) in 2μl Grace's insect medium (Gibco, Grand Island, NY.). After 6hrs, the moths were chilled to 0°C and dissected. Ovaries and the abdominal walls (primarily fat body) were collected separately for homogenization in PBS (7.4). Protein content was determined on two aliquots of each sample using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). ^{35}S methionine labeled proteins when necessary, were precipitated by trichloro-acetic acid (TCA) and were scintillation counted. Equal quantities of labeled proteins were resolved on 5 or 8.5% to 20% gradient SDS-PAGE after boiling in SDS sample buffer.

Immunoprecipitation of ^{35}S Labeled Proteins

^{35}S-Met labeled proteins were homogenized in PBS (7.2-7.4) and the 10,000xg supernatant was collected. Protein samples standardized for the amount of protein were diluted two-fold with NET --150 mM NaCl, 2mM EDTA and 50 mM Tris-HCl (7.6). Triton-X 100 was added to 1% and to this, 0.1 volumes of *Galleria* anti-YP4 antiserum were added. The mixture was incubated 16 hrs at 4°C. The antigen-antibody complexes were precipitated by addition of 0.1 volumes of protein A -Sepharose (Sigma) with continued incubation for 2 hrs. The precipitates were collected by centrifugation at 12,000xg for 10 sec and then washed three times with NET. Washed precipitates were dissolved in SDS-sample buffer, boiled and then centrifuged again before electrophoresis. After electrophoresis the slab gels were prepared for fluorography. They were soaked in

fluoroenhance (Research Product, Int. Corp, IL) for 45 minutes and then vacuum dried on a gel dryer. Subsequently, the dried gels were exposed to X-ray films that had been pre flashed with red light. They were placed at -80 °C for periods ranging from one to four weeks. The films were then developed and fixed as usual.

Organ culture of Ovaries

Day zero pupae and previtellogenic female pharate adults (eye darkened, tarsal claws not melanized - see table I) were immersed in 70% ethanol briefly and washed in sterile Ringers solution. They were dissected, in Galleria Ringer's. After the ovarian cysts (D0) or exfolliated ovarioles (D3/4) were dissected out and cleaned of any adhering fat body they were washed three times in sterile Ringers and once in Grace's insect medium. Finally the ovaries were transferred to Grace's medium containing 0.5% fetal calf serum fraction 5, 50ug/ml gentamycin in Graces medium (Gibco BRL) and ecdysone 3-5 ug/ml. Two to three ovaries were cocultured. The cultures were placed in a 100% oxygen atmosphere and incubated at room temperature with gentle shaking. Every 20 to 24 hrs. the culture medium was replaced with fresh preparation.

Ovarian transplantation

Ovarian cysts were removed from surface sterilized day seven (D7)last instar larvae that weighed above 250mg on day five(D5). Last instar larvae that weighed below 240mg (potential male) on D5 were used for ovary implantation on D7. A single ovary was implanted into the hemocoel of a last instar male larva that was starved for two hours, through surgical incision dorsally, just lateral to the middorsal line. A mixture of crystalline penicillin/ streptomycin/ phenyl thiourea (1:1:1) was then placed in the wound to prevent infection and the wound was sealed with bee wax. The hosts were

allowed to pupate and continue adult development. When they emerged as adults the ovaries were removed by dissection and frozen immediately in liquid nitrogen for RNA or protein analysis.

RESULTS

Isolation of *G. mellonella* Ovariole-specific cDNA clones

In order to isolate DNA sequences of developmentally regulated female-specific genes such as those encoding yolk protein from the ovary, a cDNA library was prepared starting with poly A+ RNA isolated from vitellogenic ovarioles of day eight (D8) pharate adult. Since many different classes of mRNA sequences including a plethora of house keeping genes would be represented in the cDNA library and since the yolk proteins are female specific, the library was differentially screened with single strand cDNA probes derived from male pharate adults [(-)ve probe] and from vitellogenic ovarioles [(+)ve probe]. From an initial screening of approximately 20, 000 plaques, 78 putative female/ovariole-specific clones (fig. 2) were isolated. After a second differential screen of these 78 clones (fig.2.) with (+)ve and (-)ve probes, 45 of these clones were confirmed to be female specific. Screening of these clones with cDNA derived from ovariectomized pharate adults as the (-)ve probes and ovariole- derived cDNA as the (+)ve probe, showed that 36 clones were ovariole-specific. The number of clones and probes used in each screen is given in table 3. Recombinant lambda DNA was prepared from nine of the 36 putative ovariole specific cDNA clones and was digested with EcoR I to release the cDNA inserts. The sizes of the inserts shown in figures.3i and 3ii show that clones Ov # 6, 7, 11, 15, 23 and 28, had single 1.6, 0.7, 0.9, 1.2, 2.0 and 0.3 kb inserts (fig.3i) while clones Ov #3, 4 and 5 had more than one insert (fig.3ii, panel A). Clones # 3 and 4 had three inserts (2.9, 1.0 and 0.7 kb; 1.2, 1.0 and 0.6 kb respectively) and #5 had two inserts (1.6 and 0.8 kb)}. When digested with Not 1 enzyme (which has a recognition site within the EcoR I adapter used in cDNA cloning), the same size inserts were released (figure 3b, panels A) indicating that these inserts represent tandomly ligated independent cDNAs that were cloned into the vector arms.

FIGURE- 2 Differential screening of ovariole specific cDNA library.

The autoradiograms shown are of duplicate NC filters from the second screen that were hybridized to cDNA probes derived either from ovariole (+)ve (top) or day zero male (-)ve (bottom) pupal poly A+ RNAs. Hybridization was done overnight, in 5x Denhardt's hybridization buffer at 65°C. Final washes were in 0.2x SSC at 68°C. The filters were air dried and exposed over night without intensifying screen, at -70°C. For details please see materials and methods.

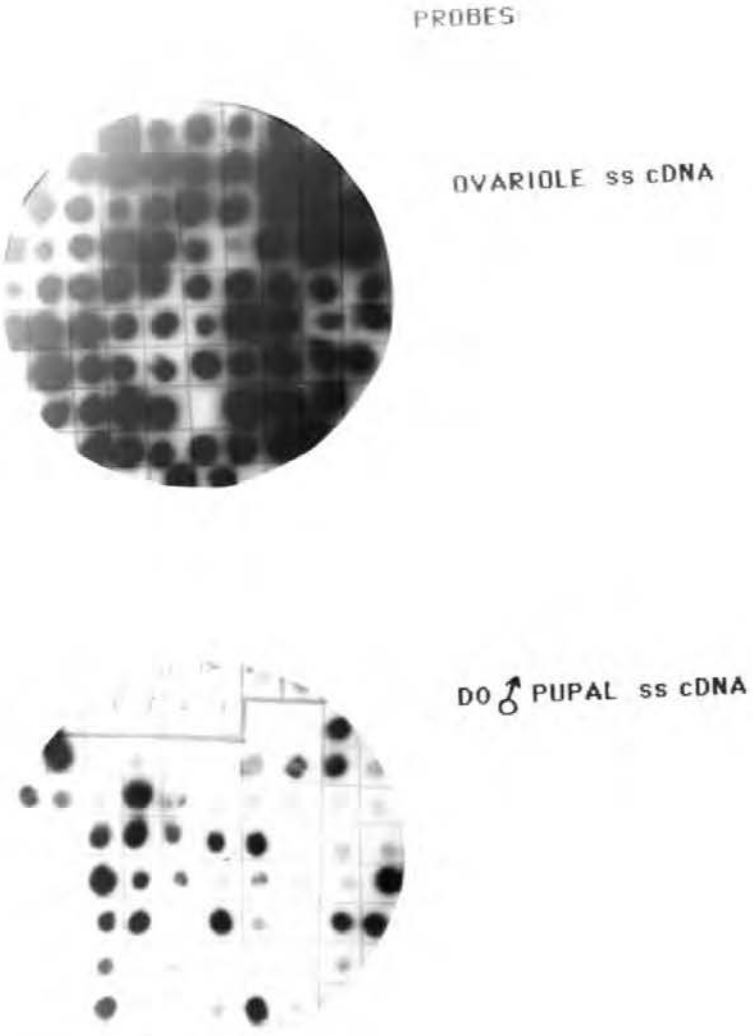
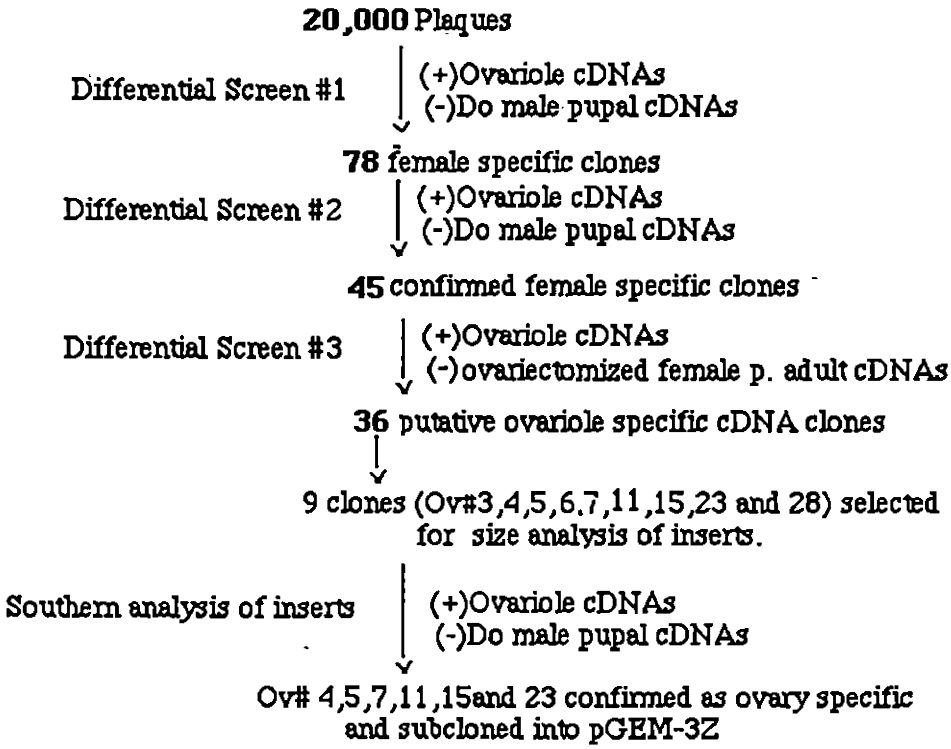


Table 3: Differential screening of ovariole library

Screening	(+)ve Probe	(-)ve Probe	Number of Clones Isolated
20,000	single strand cDNAs		
First	D8 pharate adult ovariole	D0 male pupae	78
Second			45
Third		D8 pharate ovariectomized female	36



In order to confirm ovariole specificity, cDNA inserts were electrophoresed in agarose gel and were sandwich blotted by capillary transfer onto two nylon (Zetabind) membranes. One blot was hybridized to an ovariole derived cDNA probe and the other to pharate adult male cDNA probe. The data presented in panels B and C of figure 3i) and and panel B of 3ii), show that inserts from all the clones (except for clone #6), hybridized only to the ovariole derived cDNA probe. The EcoR I inserts from clone # 3 (0.7 kb) and #15 (fig.3ii B, midpanel ; fig.3i, panel B) did not give a positive signal with ovariole probe, probably due to failure of transfer during blotting (see below). The cDNA inserts from clones #4, 5, 7, 11 and 23 (1.0, 1.6, 0.7, 0.9, 2.0 kb respectively) strongly hybridized only to the ovariole-derived probe and were therefore subcloned into pGEM-3Z (designated Ov4, 5, 7, 11, 15 and 23) for further study. Clones 3, 4 and 6 were not used in further analysis.

Cross hybridization reveals six independent groups of ovariole specific clones

The 36 ovariole-specific cDNA clones could represent messages from independent genes or could be redundant clones. Thus to identify the cross reacting clones among the 36, five of the subcloned ovariole cDNAs, 5 , 7 , 11 , 15 and 23 were used sequentially, to probe plaque lifts of the 36 ovariole specific lambda clones plated in a grid pattern. Nitrocellulose filters lifted from these plates were hybridized to the ^{32}P labeled probes prepared from subclones, Ov 5, 7, 11, 15 or 23. The data presented in Figure. 4 show that these five clones hybridized to 21 clones and the data are summarized in table-4. For example, Ov5 hybridized to clones 5, (18), 22, 25, 30,31. Only clone 18 gave a faint signal with Ov5 and very strong signal with Ov15 while all others hybridized to only a single probe.

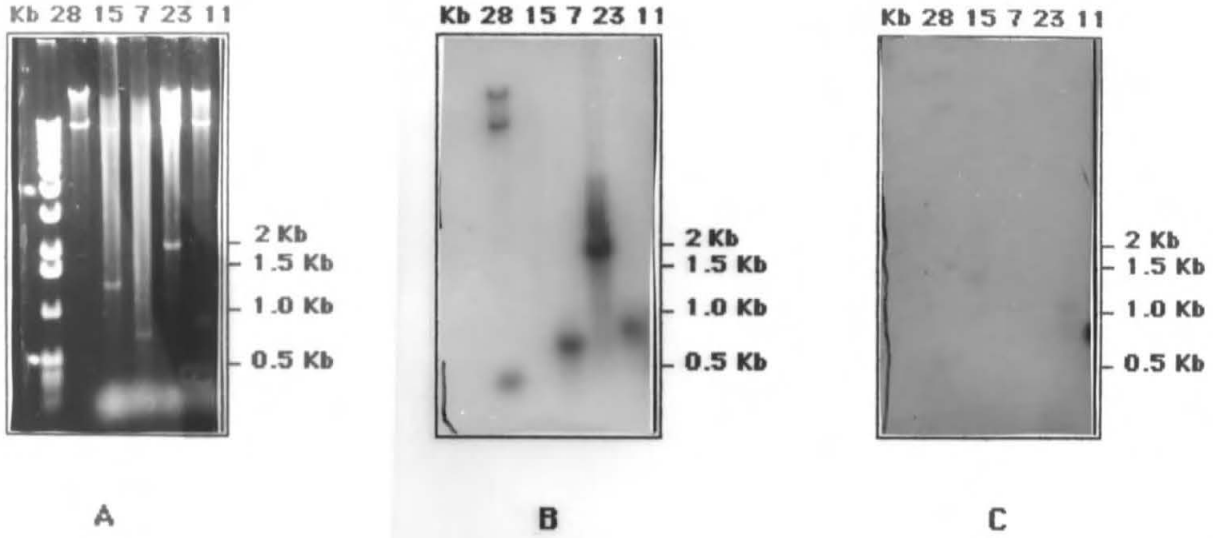
Each of the plasmid probes hybridized to a different set of lambda clones suggesting that these five cDNAs could potentially represent five different genes or non-

FIGURE- 3. Size analysis of cDNA inserts and Southern analysis to confirm their ovariole specificity

Electrophoretic separation of EcoR I digested DNA from the putative ovariole specific cDNA clones were visualized by ethidium bromide staining (figure 3. i and ii, Panels A). Autoradiograms of the blotted DNA hybridized to ^{32} PαdCTP (panels B) labeled ovariole and day zero male pupal cDNAs (panels C) and washed at 68°C. The number on top of the panels refer to cDNA clones. Figure.3 i) 28, 15, 7, 23 and 11, in lambda gt10 vector.

Kb- 1Kb DNA ladder (BRL).

Figure.3 ii) panels B and C show the Southern analysis of uncut (lanes marked -U) or EcoRI digested (lanes marked -E) cDNA clones, 3, 4 , 5, 6 and 7. The left most gel (panel A.) and autoradiograms (panels B and C) show the cDNA clones 3 and 5 digested with Not I (recognition sites for which are contained in the EcoR I adaptors ligated to both ends of the cDNAs).

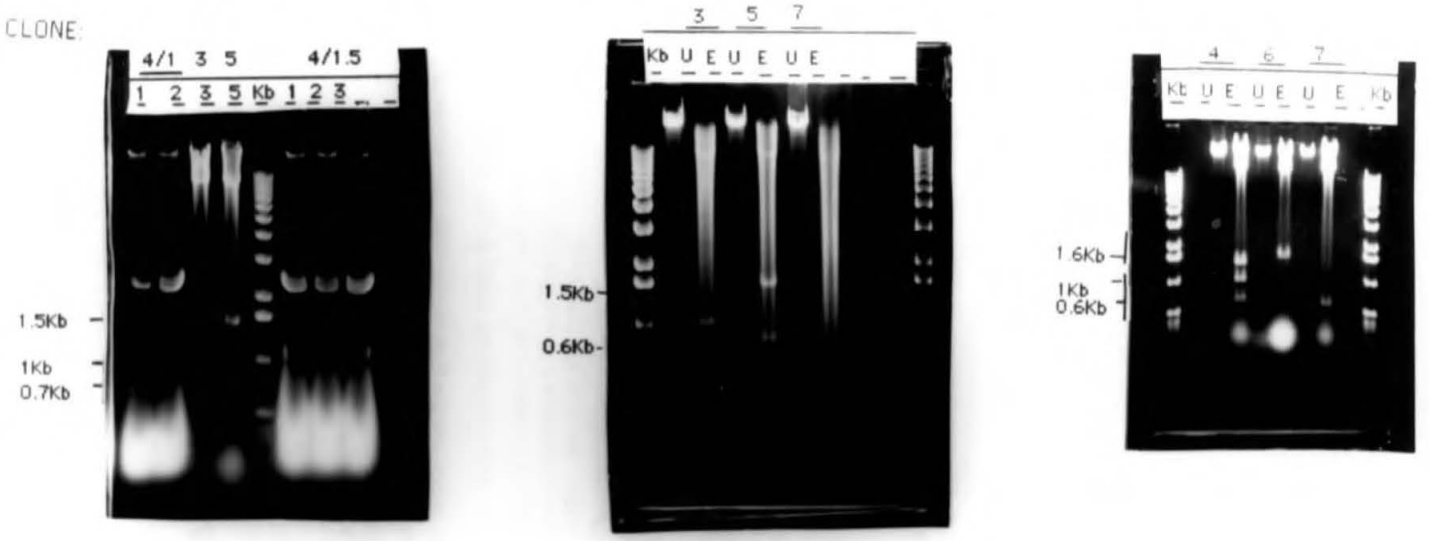

Kb 28 15 7 23 11
2 Kb
1.5 Kb
1.0 Kb
0.5 Kb
A
Kb 28 15 7 23 11
2 Kb
1.5 Kb
1.0 Kb
0.5 Kb
B
Kb 28 15 7 23 11
2 Kb
1.5 Kb
1.0 Kb
0.5 Kb
C

3 ı)

3 i i

OVARIOLE SPECIFIC CLONES

A

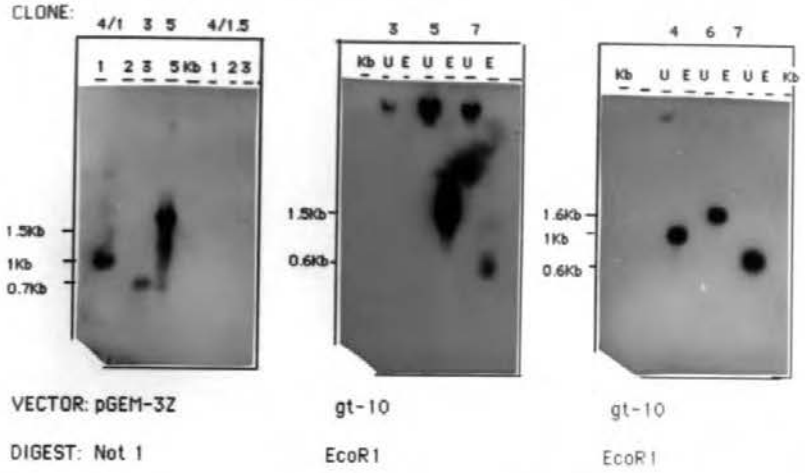


VECTOR: pGEM-3Z	λ gt-10	λgt-10
DIGEST: Not 1	EcoR1	EcoR1

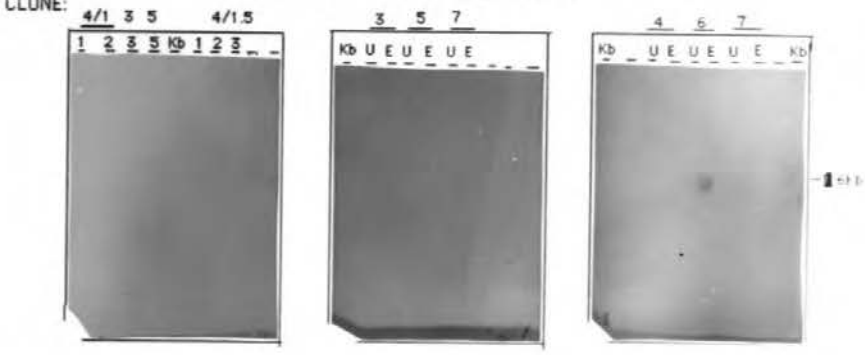
SOUTHERN SANDWICH BLOTS

OF OVARIOLE SPECIFIC CLONES

B (+) VE SCREEN (OVARIOLE SS cDNA PROBE)



C (-) VE SCREEN (DO SS cDNA PROBE)



overlapping regions of fewer genes. Fifteen lambda clones did not hybridize to any of these probes, thereby indicating the presence of cDNAs representing other genes as well. Furthermore, inserts from clones 5 and 7 hybridize to 6 and 9 respectively, more than any other probes suggesting that they represent abundant messages. Out of the six of non-cross hybridizing classes of ovariole specific clones, 5, 11 and 23 containing the largest inserts representing three classes were selected for further analysis.

Clone 18 did not grow very well and failed to produce good lysates for lambda DNA preparation. Thus to make sure that Ov5 and Ov15 do not represent different parts of the same transcript, northern analysis as was performed also with Ov15 as probe.

Ovariole Specific Expression of cDNA clones Ov 5, Ov11 and Ov23

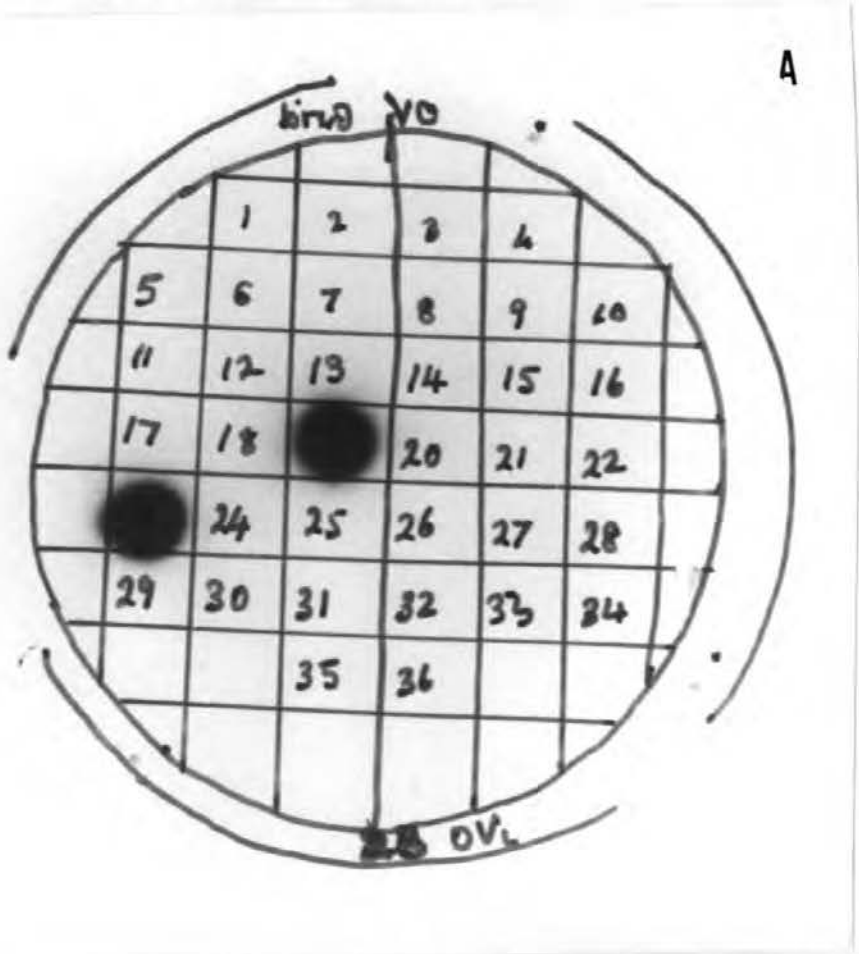
In order to confirm these cDNAs represent different ovariole specific genes by the the size of the mRNA corresponding to the cDNAs, a northern blot analysis was performed using poly A + RNA from vitellogenic ovarioles, last instar larvae of mixed sex, male pharate adults and gonadectomized female pharate adults. Plasmid clones bearing the cDNA Ov 5, Ov 11, Ov 23 and Ov15 were used as probes to hybridize to individual blots.

Data presented in figures 5a and 5b show that Ov5, Ov11,Ov23 cDNAs hybridized, predominantly to 2.0, 1.1 and 2.3 kb transcripts, respectively, present in the RNA extract from ovarioles, but not in tissues from males, last instar larvae or gonadectomized female pharate adults (Fig. 5a, lanes Ov and 5b lanes 3). Ov 15 cDNA hybridized to 1.8 kb transcripts present in the ovariole as well as in day five last instar larvae (Fig 5 b, left panel lanes 1 and 3) and hence was not further studied. The results confirmed that, Ov5, 11 and 23 cDNAs represent genes that are expressed only in the ovarioles and based on the size of their cognate transcripts these three clones appear to represent three different genes.

Since the 960 bp Ov11 cDNA hybridized to a 1.1 kb transcript, it is reasonable to assume that Ov11 contains a near full-length cDNA insert. The Ov 5 cDNA with an insert size of 1.6 kb, hybridized to 2.0 kb size transcript (Fig. 5a). In addition, it hybridized to

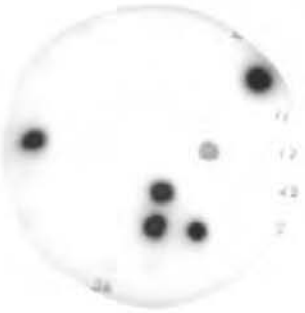
FIGURE- 4 Six classes of non- cross hybridizing ovariole specific clones represented by Ov5, 7, 11,15 and 23.

The autoradiogram of the five nitrocellulose circle filters lifted from two identical grid LB/ agarose plates containing the 36 ovariole specific plaques isolated after several rounds of screens hybridized individually to nick translated, α^{32}P-dCTP labeled, cDNAs- Ov5, 7, 11, 15 and 23 subcloned in the plasmid vector pGEM-3z. The probes as given below each grid, 5O = Ov5, 7O = Ov7, 11O = Ov11, 15O = Ov15, 23O = Ov23. The pattern of numbering the plaques (autoradiograms at the bottom are flipped left to right) and hybridization pattern of Ov23 is shown in panel A.

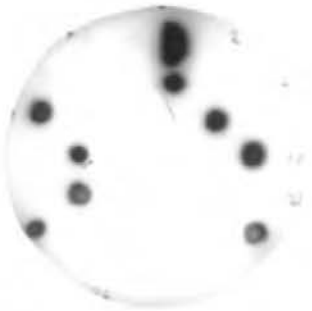


B

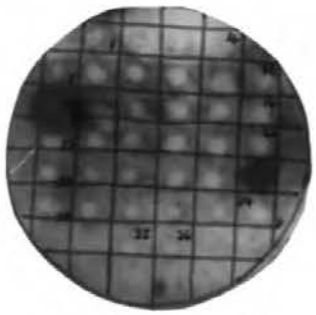
DIVIDING INTO CROSS HYBRIDIZING GROUPS
[OVARIOLE SPECIFIC CLONES(36)]



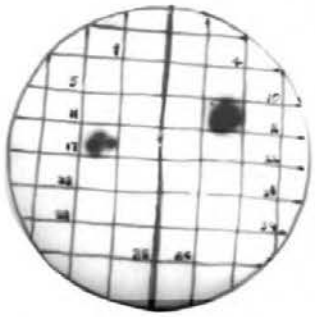
PROBE: CLONE #50(NICK TRANSLATED).
SIZE: 1.5 Kb



PROBE CLONE #70 (NICK TRANSLATED)
SIZE 0.7 Kb



PROBE: CLONE #11 0 (NICK TRANSLATED)
SIZE: 0.9 Kb



PROBE CLONE #15D(NICK TRANSLATED
SIZE 1.2 Kb

Table 4: Cross Hybridizing Groups

Probe clone	Cross Hybridizing Clones								
5 Ov		5	18	22	25	30	31		
7 Ov	2	7	12	16	17	21	27	37	
11 Ov		11	28						
15 Ov		15	18						
23 Ov		19	23						

a less intense band of ~ 4.0 kb. This could represent products of alternatively spliced transcripts or transcripts of cross hybridizing genes. Faint bands of larger size, ~3.0 and ~7.5 kb also appeared in the ovariole RNA probed with Ov 11 and 23. Now that these clones were confirmed to represent different ovariole specific genes, they were further studied. While Ov5 and Ov23 cDNAs are not full length, it is still possible that they contain the entire coding sequence.

Developmental stage specificity of expression of Ov5, Ov11 and Ov23

Each of the vitellogenic moth ovarioles that was used in the previous northern analysis contained all oogenic stages from S1 to S11, ie., from initiation of oogenesis to initiation of choriogenesis. Thus, the transcripts identified could have been from the previtellogenic, vitellogenic or post-vitellogenic follicles.

Therefore, RNA was extracted from pre-vitellogenic (S1- S3), early vitellogenic (S5- S6), late vitellogenic (S7-S9), and choriogenic stage (S11 - S12) egg follicles dissected from ovarioles. The dimensions of the follicles representing the different stages were measured to facilitate reproducible staging of the follicles (Table.2). In addition, 1-2 day old oviposited eggs were also examined in order to see if any of the messages for these ovariole cDNAs persist in the embryo.

The autoradiograms of northern blots of staged follicle poly A+ RNAs are presented in figure 6, showing that Ov11 and Ov5 transcripts are present only in the vitellogenic S5 - S9 follicles but not in pre-vitellogenic and choriogenic stage egg follicles or in embryos. However, in the pre-vitellogenic ovariole, a larger (2.0 kb) transcript of very low intensity is seen in the blot hybridized to Ov11 (arrow head in Fig. 6, lane 1). In the blots probed with Ov 5, larger ~4kb and $\geq$ 6.5kb size transcripts of low intensity are seen (fig.5a) during vitellogenic stages (lanes 2 and 3).

FIGURE 5a: Northern blot analysis showing ovariole specificity and transcript size of cDNAs Ov5 and Ov11.

Autoradiograms of northern blots with 1.5 ug poly A+ RNA each, extracted from, (D5 L.I)- day five last instar larvae, (O-) male pharate adult, (OvL) ovarioles and (- OvL) female pharate adults minus ovarioles, hybridized to nick translated cDNA probe as indicated below each panel. The numbers on the side of the panel are the sizes of the RNA molecular weight markers (BRL). The arrows indicate the cross hybridizing low abundant messages.

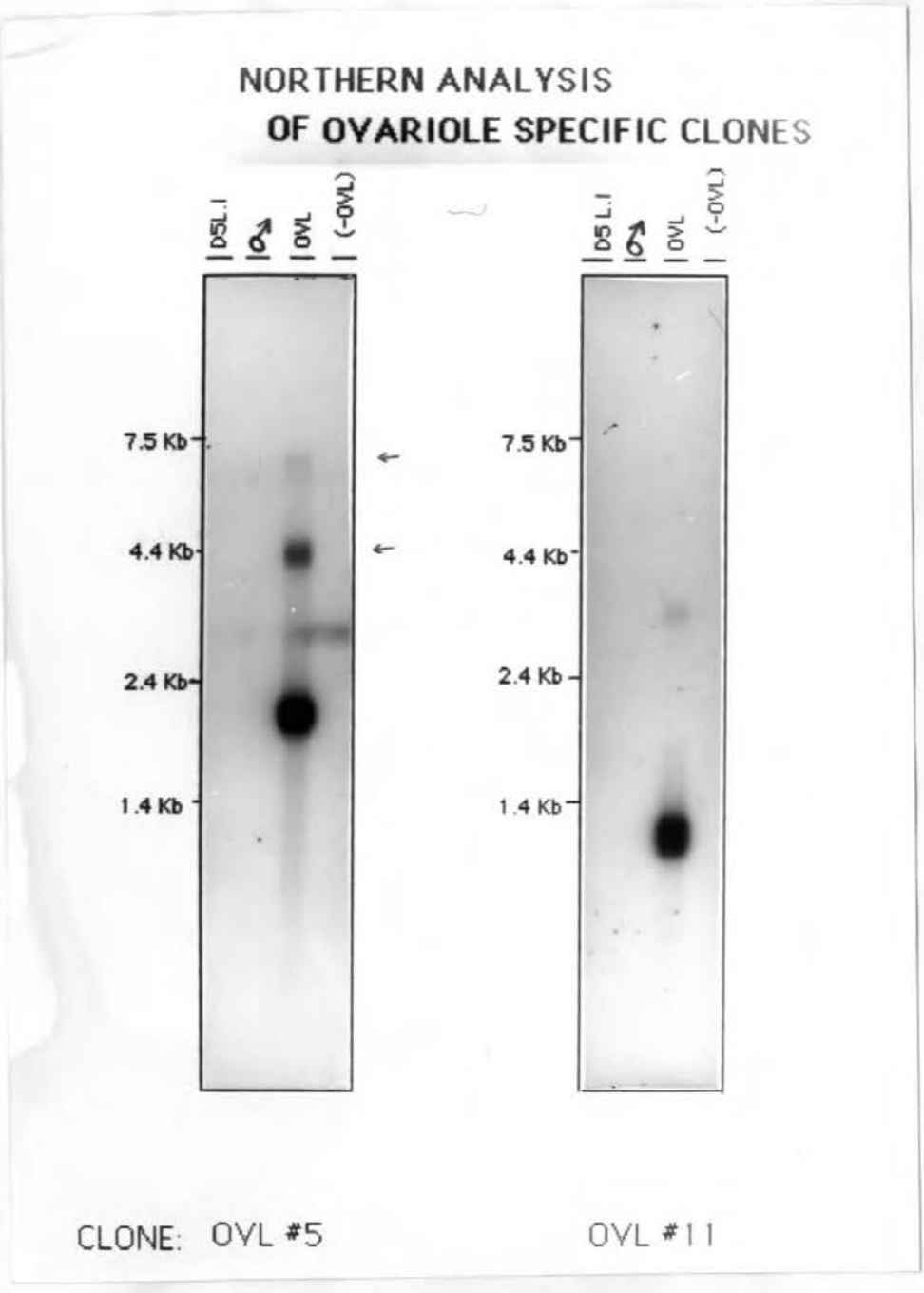
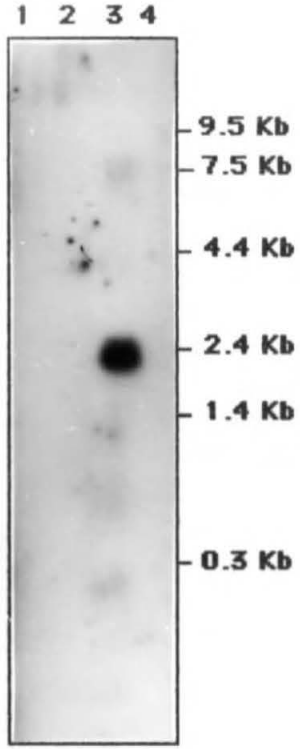
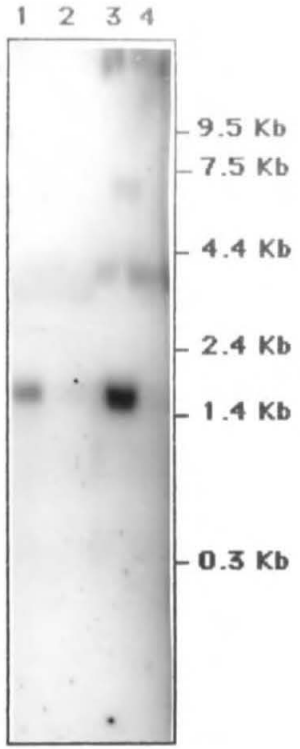

NORTHERN ANALYSIS
OF OVARIOLE SPECIFIC CLONES
D5L.I
♂
OVL
(-OVL)
7.5 Kb
4.4 Kb
2.4 Kb
1.4 Kb
D5 L.I
♂
OVL
(-OVL)
7.5 Kb
4.4 Kb
2.4 Kb
1.4 Kb
CLONE: OVL #5
OVL #11

FIGURE 5 b: Northern blot analysis showing ovariole specificity and transcript size of cDNAs N23 (Ov23) and Ov 15.

Autoradiograms of northern blots containing 2. 0 ug poly A+ RNA each, extracted from 1) day five last instar larvae, 2) male pharate adult, 3) ovarioles and 4) female pharate adults minus ovarioles that were hybridized to α^{32}P-dTTP labeled cDNA probe as indicated below each blot. The numbers on the side of each panel are the sizes of the RNA molecular weight markers (BRL).


1 2 3 4
9.5 Kb
7.5 Kb
4.4 Kb
2.4 Kb
1.4 Kb
0.3 Kb

N 23


1 2 3 4
9.5 Kb
7.5 Kb
4.4 Kb
2.4 Kb
1.4 Kb
0.3 Kb

Ov 15

Ov23 showed a different developmental expression pattern than Ov5 ans Ov11, hybridizing to 2.3 kb transcripts in follicles of all oogenic stages (fig. 7, lanes 1,2,3 and 4). In addition, Ov23 messages were also present in the early embryos (fig. 7 , lane 5). Apparently lower levels of the message during the later stages of oogenesis may be due to dilution effect resulting from the accumulation of abundant stage specific messages. These results from the developmental northern analyses suggest that cDNA clones Ov11 and/ or Ov5 represent transcripts that are accumulated in the ovary only during the vitellogenic stages whereas Ov23 transcript is present throughout oogenesis and probably expressed as long lived mRNAs early in oogenesis that persist through embryogenesis.

In Situ Hybridization Analysis of Ov5, Ov11 and Ov23 Transcripts

Since egg follicles of polytrophic ovaries such as those of the wax moth are composed of three major cell types; the single cell layer of follicle cells, surrounding germ line derived oocyte and seven nurse cells, the ovary specific transcripts hybridized to Ov5, 11 and 23 could be from any one of these cell types. *In situ* hybridization of whole fixed ovarioles was carried out as described in materials and methods, using digoxigenin labeled cDNA probes to identify the specific cell type in which the Ov5, 11 and 23 transcripts are produced.

cDNA inserts were digoxigenin-dUTP labeled by random priming and were hybridized to whole fixed ovarioles. The hybridization of probes to the mRNAs, was detected by the red color precipitate produced by the peroxidase reaction with its substrate diamino benzaminidine (DAB).

In control preparations in which digoxigenin -dUTP labeled cDNA probe was not added, none of the cells in the follicles showed the colored reaction product (Fig.8D). Similarly, fat body cells processed with digoxigenin labeled Ov11 or Ov23 cDNA were also negative (data not shown) suggesting that the positive color reaction observed in the cells is due to specific hybridization of the digoxigenin labeled cDNAs.

Figure 6: Developmental northern analysis of Ov5 and Ov11 cDNA

Autoradiograms of northern blots of 0.3 μg poly A+ RNA extracted from 1) pre-vitellogenic 2) early vitellogenic 3) late vitellogenic 4) chorionic egg follicles and from 5) early embryos (1 to 2 day old) hybridized with nick-translated α^{32}P-dCTP labeled Ov11 or Ov5 (F20) cDNA probes as indicated below the panel. The faint bands of low intensity are indicated by arrows. The numbers on the left of the autoradiogram indicate the size of the RNA ladder markers.

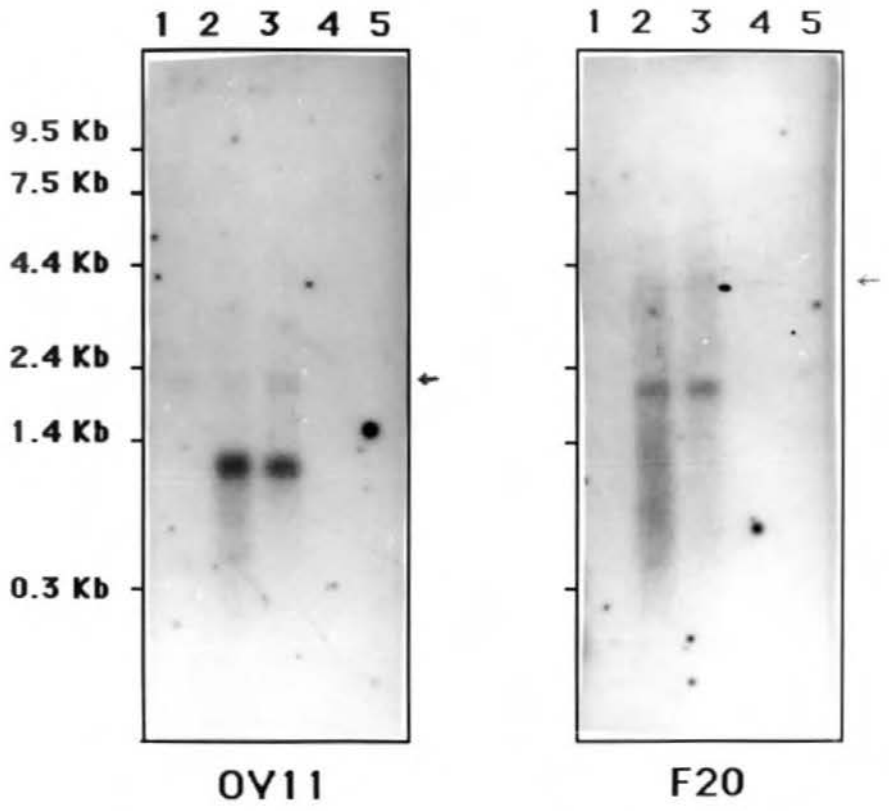
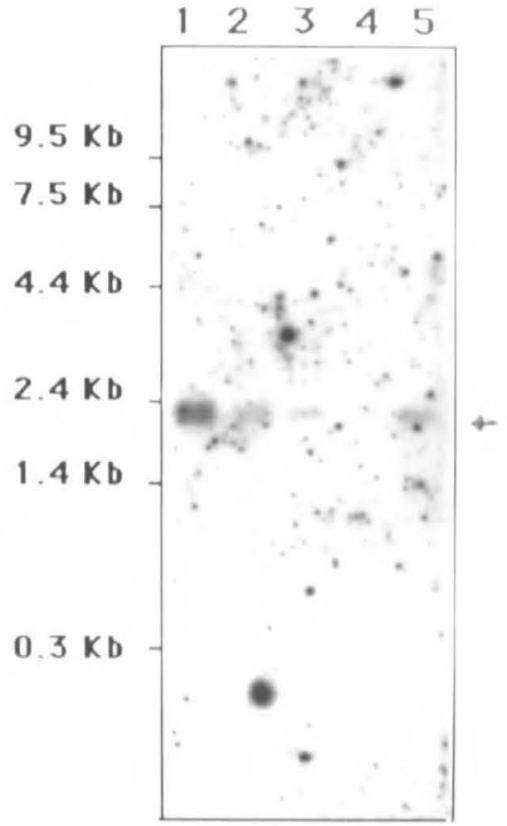

1 2 3 4 5
9.5 Kb
7.5 Kb
4.4 Kb
2.4 Kb
1.4 Kb
0.3 Kb
OV11
1 2 3 4 5
F20

Figure 7: Developmental northern analysis of Ov 23 cDNA

Autoradiograms of northern blots of 0.6 μg poly A+ RNA extracted from 1) pre-vitellogenic 2) early vitellogenic 3) late vitellogenic 4) chorionic egg follicles and from 5) early embryos (1 to 2 day old) hybridized with nick-translated α^{32}P-dCTP labeled Ov23 cDNA probe. The 2.3 kb transcript is marked by an arrow. The numbers on the left of the autoradiogram indicate the size of the RNA ladder markers.



N23

In situ hybridization with Ov11 and Ov 5 cDNA probes are shown in Figures 8a and 8b. Only the follicle cell layer surrounding the oocyte showed hybridization. The nurse cells, follicle cells covering the nurse cell cap and the oocytes were negative (colorless). The color reaction appeared uniform in intensity in the follicle cell layer. The vitellogenic egg follicles of 80μm to 250μm showed the most intense color reaction. The oocytes did not show color reaction when the follicle cell layer was removed by dissection. The S9-follicles in which the nurse cells are disintegrating show lower levels of hybridization to Ov11 probe. The results show that Ov11 and Ov5 genes are expressed only in the follicle cells of the vitellogenic ovaries; therefore Ov 5 cDNA was renamed F20, to indicate its follicle cell specificity and transcript size, and Ov11 cDNA was renamed F11.

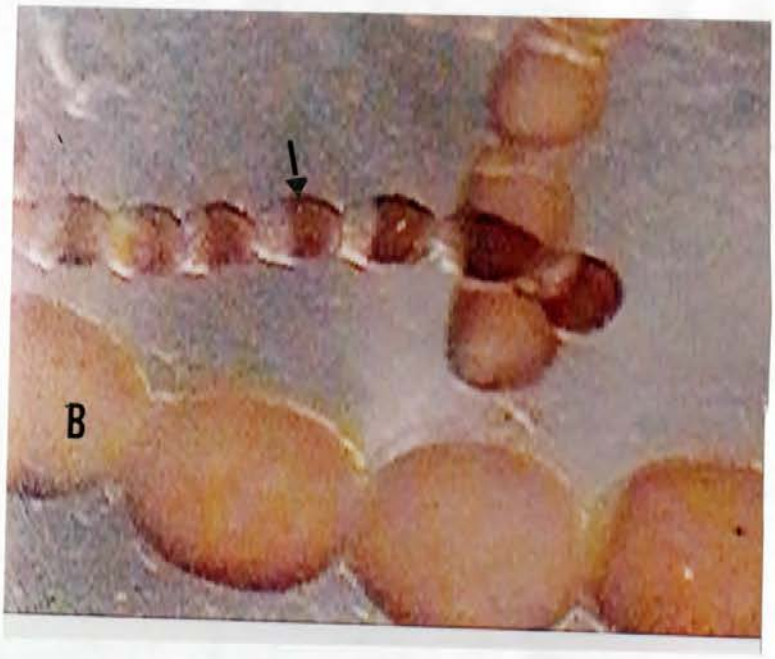
In contrast to F20 (Ov5) and F11 (Ov11) cDNA, Ov23 probe hybridized strongly and specifically to nurse cells (Fig.8C). Reaction product was uniformly distributed within the nurse cells. By virtue of their cell specificity, the positive color reactions with Ov23, F20 and F11 serve as good positive controls for each other in the *in situ* hybridization studies. The positive reaction to Ov23 was seen in nurse cells early in oogenesis, very strongly in previtellogenic follicles (S3- S4), and appear to persist in nurse cells up to stage 9 when all the nurse cell contents are being transported to oocyte. The oocyte from stage 9, continued to show diffused color reaction product. More importantly, northern blot data reported in the previous section clearly shows that the transcripts are present in mature oocytes and 1-2 day old embryos, suggesting that Ov 23 cDNA is a nurse cell derived maternal message. Based on the cell source and the transcript size, Ov23 was renamed N23, identifying its nurse cell specificity and 2.3 kb size transcript.

I next concentrated on characterizing cDNA clones F11 and F20, since they showed the expression pattern expected of the follicle-specific yolk polypeptides.

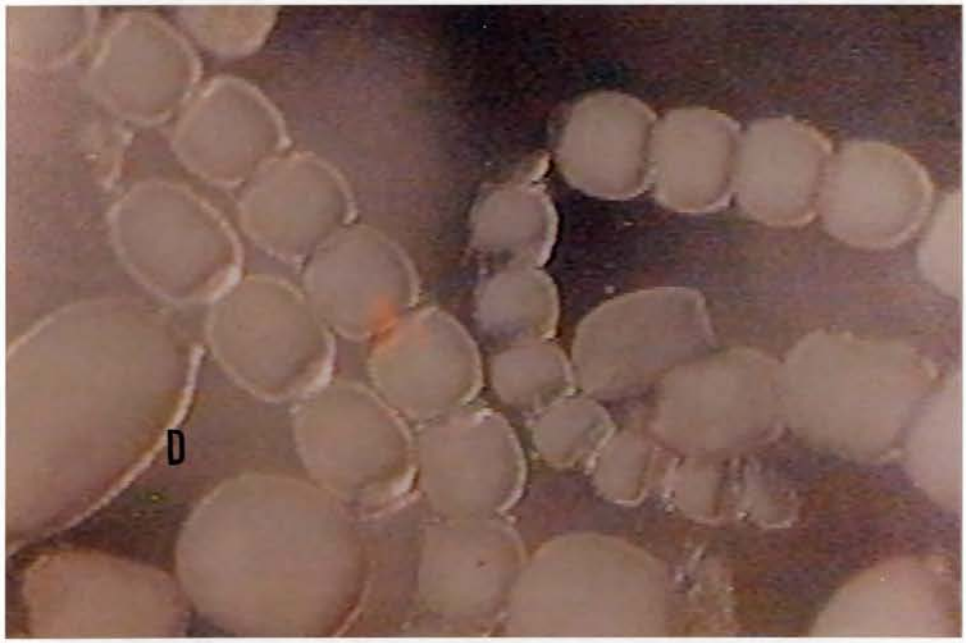
FIGURE 8: In situ hybridization Ov5 Ov11 and Ov23 digoxigenin probes to ovarioles.

In panel-A, digoxigenin-dUTP labeled Ov5 cDNA insert hybridizing to the follicle cells surrounding stage- 7 (0.25mm) egg follicles is shown. Similarly panel-B shows hybridization of Ov11 cDNA probe to follicles of stages- 5 (0.15mm) to 9 (0.3 mm). Note the clear colorless area surrounding the nurse cells and the nurse cells also do not hybridize. Panel-C), hybridization of Ov23 probe to nurse cells of the same stage egg follicles as in panel A. The control reaction of fixed ovarioles, that had been carried through all the steps except for the addition of a labeled probe is shown in panel D.


A


B


C
J


D

Identification of follicle specific Yolk polypeptide-encoding cDNAs

Nucleotide sequencing of F11 cDNA

To enable identification of the polypeptides encoded by the vitellogenic stage follicle cell specific F11 and F20 cDNAs, the insert DNAs subcloned in the pGEM-3Z plasmid vector were sequenced completely using the dideoxy sequencing method of Sanger (1977). The sequencing strategy of cDNA F11 is shown in Figure 9a and the sequence is shown in fig. 10.

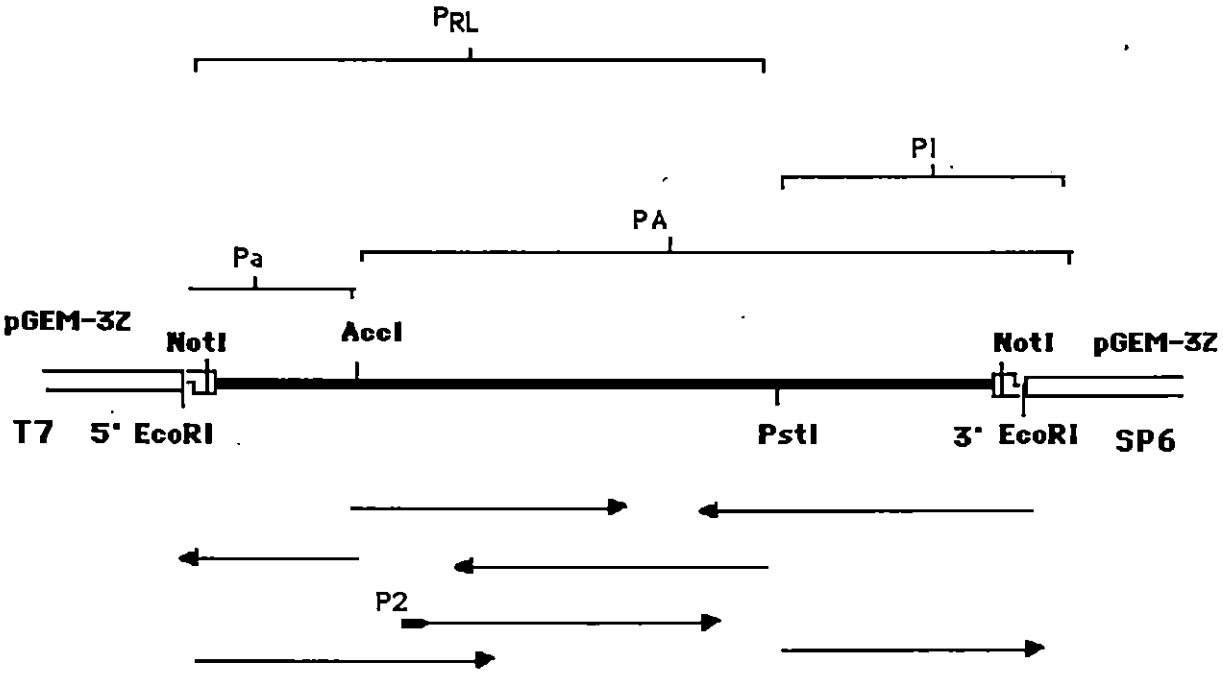
In the case of F11 cDNA (figure 9a), the single Acc1 and Pst1 sites present at 264 bp and 705 bp were utilized for subcloning. The vector pGEM-3Z, also contains an Acc I and a PstI restriction site in the 3' end (SP6 end) of the multicloning site. The F11 cDNA clone was digested with Pst1 and the small fragment of 267bp designated PI was subcloned into the Pst1 site of pGEM-3Z. The larger fragment still attached at the T7 end of the vector was religated to Pst1 site on the vector and designated PRL. Similarly, the Acc1 subclone Pa was generated with the 264 Bp T7 end piece. The larger Acc I fragment was subcloned into Acc I site of the vector to generate subclone PA. Subclones PRL, PI, PA and Pa were sequenced in both directions using the SP6 and T7 universal primers. In addition, an oligonucleotide primer P2 was also used.

F11 cDNA has the entire coding sequence

In the 952 bp F11 cDNA, a single open reading frame (Fig.10) extends from the 5' end of the cDNA. Since the transcription start site is not known, the first nucleotide of the F11 cDNA was numbered +1. The ATG present at nucleotide +4 is the likely translation initiation codon (Kozak, 1990). In addition, this start codon also follows 3 A residues that are probably part of the consensus sequence (C/AAAC/A) for translation initiation, which is proposed for genes of *Drosophila* (Cavener. D R, 1987). A poly A signal sequence AATAAA was located at +935 bp, 73 bp downstream of the stop codon TGA at

FIGURE 9 A: Sequencing strategy of F 11 cDNA.

The restriction sites Acc I and Pst I were used to generate the subclones, Pa (5' of Acc I), PA (3' of Acc I), P_{RL} (5' of PstI) and PI (3' of PstI). These subclones were sequenced in both directions using the T7 and SP6 primers from the pGEM-3Z vector ends. See text for other details of the subclones. In addition an internal oligonucleotide primer P2 (5'-GAG AGC AAG TAT CTC CAG G -3') from 304bp to 323bp was also used. Each arrow indicates the directions of sequencing and the extent of sequence data obtained from at least two sequencing trials.

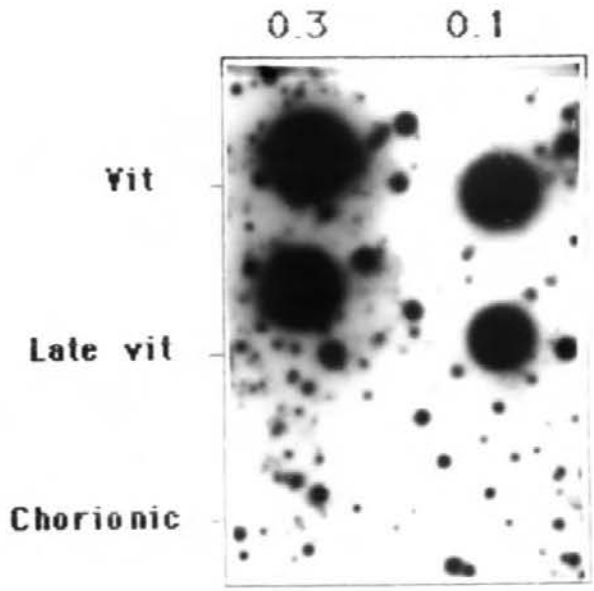

PRL
PI
PA
Pa
pGEM-3Z
NotI
AccI
NotI
pGEM-3Z
T7
5' EcoRI
PstI
3' EcoRI
SP6
P2

+862 as is typical for eukaryotes (Proudfoot and Brownlee, 1976). Since, the 3' end of the cDNA did not bear any poly A tract the cDNA source of this clone must have initiated reverse transcription close to the 3' end of the transcript. Considering the size of the transcript and allowing for the presence of a poly-A tail of about 100 bp on most eukaryotic mRNAs, the 5' end of the cDNA appears to be very close to the 5' end of the transcript. Thus, the F11 cDNA appears to code for a 286 amino acid polypeptide with a calculated molecular weight of 31.9 kDa.

The orientation of the coding strand suggested by applying the Kozak's rules for translation initiation, and by appropriately located canonical poly A+ signal sequence was confirmed also by hybridization of SP6 or T7 RNA polymerase generated sense and antisense RNA probes to ovariole RNA. Dot blots were prepared with ovariole poly A^+ RNA obtained from vitellogenic and choriogenic egg follicles. The blots were hybridized individually to probe transcripts produced in either direction from the F11 cDNA cloned in the transcription plasmid vector pGEM-3Z. Only the RNA probe transcribed by SP6 RNA polymerase (Fig.9 B) which is complementary to the T7 end 5' strand, hybridized to the message confirming the orientation of the cDNA and the coding strand inferred from the nucleotide sequence information (Figure 10).

FIGURE 9B: Identification of the coding strand by dot blot analyses with antisense RNA probe

Poly A+ RNA from ~ 60 follicles of 1) vitellogenic 2) late vitellogenic 3) choriogenic stages were diluted and denatured in formamide, spotted on to Nytran membrane and fixed by baking. The blot was hybridized to ^{32}P-CTP labeled, single strand RNA probes transcribed in vitro with SP6 RNA-polymerase from linearized template P_{RL} (the large Pst I religated fragment) subclone. The RNA sources are indicated at the left hand side and the dilutions are indicated above the figure.

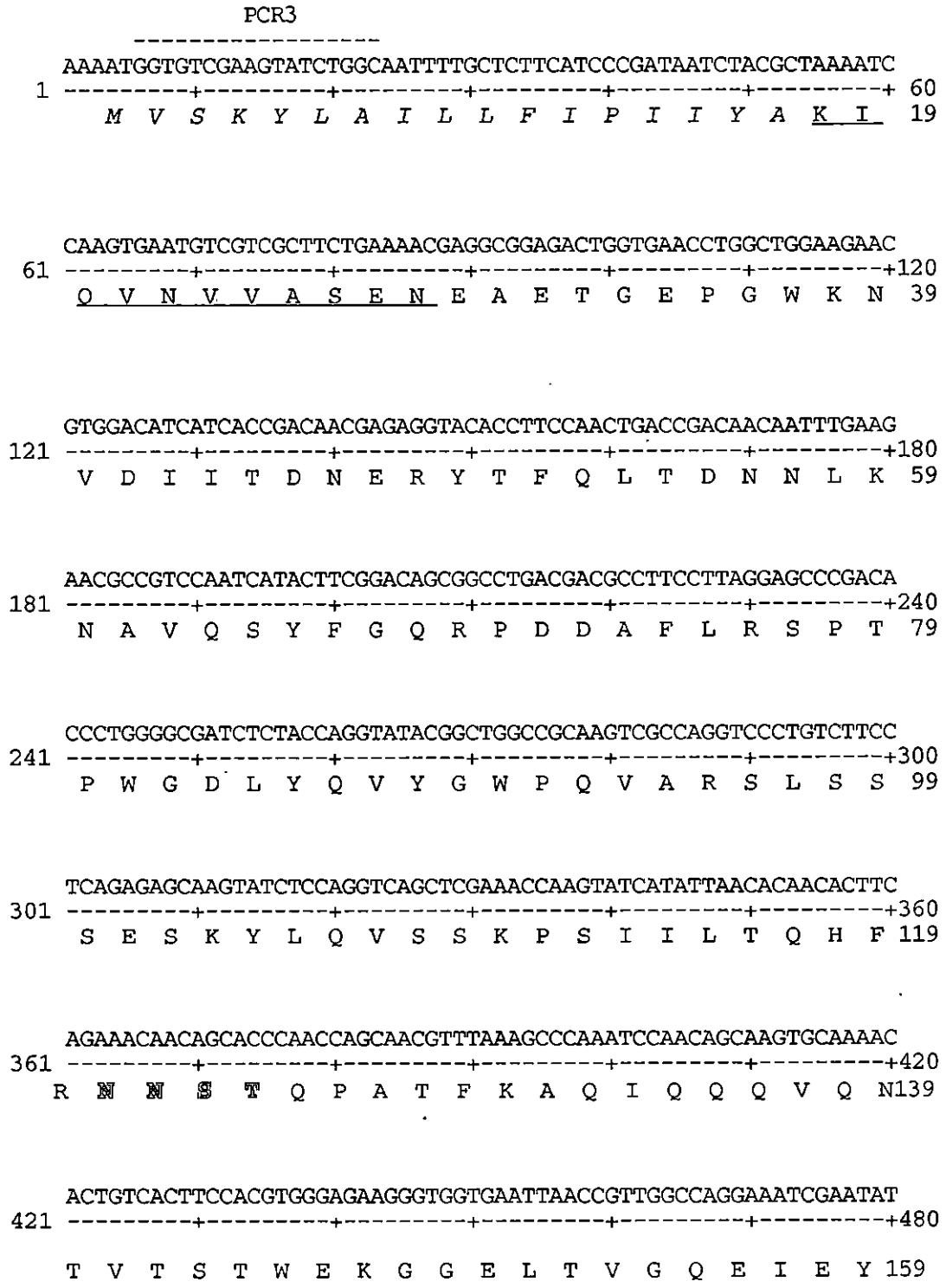


F 11

(Antisense probe)

FIGURE 10: Complete reading frame, nucleotide and deduced amino acid sequence of F11 cDNA.

The nucleotides are numbered from the very 5' end adenine, of the cDNA starting with + 1. The translation start codon AUG is at +4. The polyadenylation signal at +932bp is underlined and the stop codon at +862bp, identified with an asterisk. The lower set of numbers on the right side indicate the deduced amino acid position. The two adjacent potential N-glycosylation site are marked by bold letters. The serine, threonine rich region (for potential phosphorylation) of the polypeptide, are identified by italics. The doted lines above the nucleotide sequence, marked PCR3 and PCR5 are the primer sequences used in PCR analysis of genomic DNA (described in a subsequent section).


PCR3
AAAATGGTGTCGAAGTATCTGGCAATTTTGCTCTTCATCCCGATAATCTACGCTAAAATC
1 ---------+---------+---------+---------+---------+---------+ 60
M V S K Y L A I L L F I P I I Y A K I 19
CAAGTGAATGTCGTCGCTTCTGAAAACGAGGCGGAGACTGGTGAACCTGGCTGGAAGAAC
61 ---------+---------+---------+---------+---------+---------+120
Q V N V V A S E N E A E T G E P G W K N 39
GTGGACATCATCACCGACAACGAGAGGTACACCTTCCAACTGACCGACAACAATTTGAAG
121 ---------+---------+---------+---------+---------+---------+180
V D I I T D N E R Y T F Q L T D N N L K 59
AACGCCGTCCAATCATACTTCGGACAGCGGCCTGACGACGCCTTCCTTAGGAGCCCGACA
181 ---------+---------+---------+---------+---------+---------+240
N A V Q S Y F G Q R P D D A F L R S P T 79
CCCTGGGGCGATCTCTACCAGGTATACGGCTGGCCGCAAGTCGCCAGGTCCCTGTCTTCC
241 ---------+---------+---------+---------+---------+---------+300
P W G D L Y Q V Y G W P Q V A R S L S S 99
TCAGAGAGCAAGTATCTCCAGGTCAGCTCGAAACCAAGTATCATATTAACACAACACTTC
301 ---------+---------+---------+---------+---------+---------+360
S E S K Y L Q V S S K P S I I L T Q H F 119
AGAAACAACAGCACCCAACCAGCAACGTTTAAAGCCCAAATCCAACAGCAAGTGCAAAAC
361 ---------+---------+---------+---------+---------+---------+420
R N N S T Q P A T F K A Q I Q Q Q V Q N139
ACTGTCACTTCCACGTGGGAGAAGGGTGGTGAATTAACCGTTGGCCAGGAAATCGAATAT
421 ---------+---------+---------+---------+---------+---------+480
T V T S T W E K G G E L T V G Q E I E Y 159

```
   GGCTTCGACATCAAAGTGGTGTCTGTCGGTGGCAAGACCAGCTTCTCTTACACATCCAGA
481 ---------+---------+---------+---------+---------+---------+540
     G  F  D  I  K  V  V  S  V  G  G  K  T  S  F  S  Y  T  S  R 179

   TGGGGAGAAAGTGTGTCCAAATCGGAAACAGTGACCGTGGGCTCCGAATCAGGAGTTGAA
541 ---------+---------+---------+---------+---------+---------+600
     W  G  E  S  V  S  K  S  E  T  V  T  V  G  S  E  S  G  V  E 199

   ATCACCTTGGAGCCTGGCCAGGCTATTATAGCTGAACTCTTAGCGACCCGTGGCACCATG
601 ---------+---------+---------+---------+---------+---------+660
     I  T  L  E  P  G  Q  A  I  I  A  E  L  L  A  T  R  G  T  M 219

   GAGATCCAGGTAGATTATGAGGCTACTCTGTCCGGTGCTACTGCAGTTAACTACGCGAAC
661 ---------+---------+---------+---------+---------+---------+720
     E  I  Q  V  D  Y  E  A  T  L  S  G  A  T  A  V  N  Y  A  N 239

   ACCTTCAAGGGTCATCATCACTTCTGGGCGTCTGGACATCAATGCTGTGATGACGTCTGG
721 ---------+---------+---------+---------+---------+---------+780
     T  F  K  G  H  H  H  F  W  A  S  G  H  Q  C  C  D  D  V  W 259

   AGACTTGAACCCCACCGCTCACTCCCGGGAAGTTATCAAGCTAGGTTACTACTCCAGCTC
781 ---------+---------+---------+---------+---------+---------+840
     R  L  E  P  H  R  S  L  P  G  S  Y  Q  A  R  L  L  L  Q  L 279

                                  PCR 5
                              ------------------
   TCACGTTGTGATCAGAGATGCTGAGAACAACAATGTACTGTACTCTTTACCTATGAATAT
841 ---------+---------+---------+---------+---------+---------+900
     S  R  C  D  Q  R  C  *                                      286

   TTTCTAAATATTGTGTAAGTATATAATTATTGTGAATAAATAGCTATTTTCA
901 ---------+---------+---------+---------+---------+-- 952
```

YP4 Protein Expression

The size of the transcript, vitellogenic stage and follicle cell specificity of expression as well as the predicted size of the encoded protein, suggest strongly that the F11 cDNA codes for the 37 kDa follicle specific yolk polypeptide YP4, expressed in the ovarioles of *Galleria* (Shirk, 1987).

I confirmed that the yolk polypeptide YP4 is synthesized only during vitellogenesis in the ovarioles. Day 6 and day 8 pharate adult males and females were labeled with ^{35}S-Methionine for 21 hrs and 6 hrs, respectively. Their fat body, hemolymph and ovariole proteins were analyzed on 5-20% gradient SDS / polyacrylamide gels. In addition D5 pharate adult females were also labeled and their ovariole proteins were analyzed. As the data in figure 11 show, YP4 was not labeled in the fat body and hemolymph of male (fig.11a, lanes 3 and fig.11b, lane 1) or female (fig.11a, lane5 and fig.11b lane 2). As seen in fig.11a lane 7, YP4 (37 kDa) is found accumulated in the day six ovarioles, while it is barely seen in day 5 ovariole (fig.11a, lane 1).

Since presence of labeled YP4 in ovariole extracts does not prove that it is synthesized in the ovariole, polyclonal antisera raised against gel-purified YP4 was used to confirm that ovariole is the source of YP4. When ^{35}S-methionine labeled day 8 male and female fat body and ovariole proteins were incubated with the YP4 antisera, only the ovariole protein yielded the YP4 precipitate (fig.11c, lane 4) where as male or female fat body did not (fig.11c, lanes 1 and 2) confirming the ovariole specificity of YP4.

FIGURE 11a&b: SDS-PAGE analysis of ^{35}S -methionine labeled ovariole and fat body proteins from male and female adult *Galleria* .

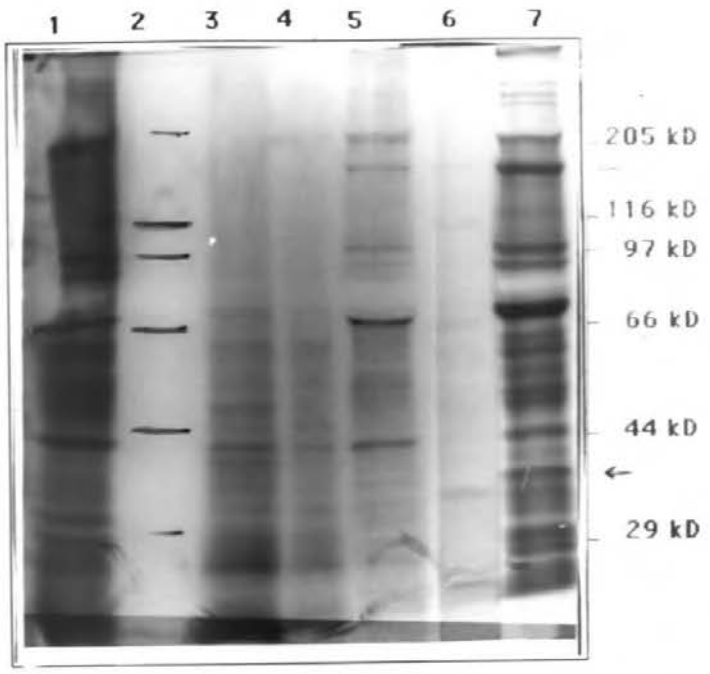
An autoradiogram of 5-20% gradient SDS-PAGE analysis of ^{35}S -methionine labeled polypeptides from the fat body, (F.B) hemolymph (H.L) the ovarioles (OvL). ~100-50µg proteins/lane were loaded in each sets of ovariole (Lanes 1 and 7), fat body (lanes 3 and 5) and H.L (lanes 4 and 6) The size of the molecular weight markers shown in lane 2. on the right.

Panel a) Lanes - 1) D5 OvL, 2) Molecular weight markers (M.W), 3) D6 male F.B, 4) male H.L, 5) D6 female F.B, 6) female H.L and 7) D6 OvL. Arrow indicates YP4 band.

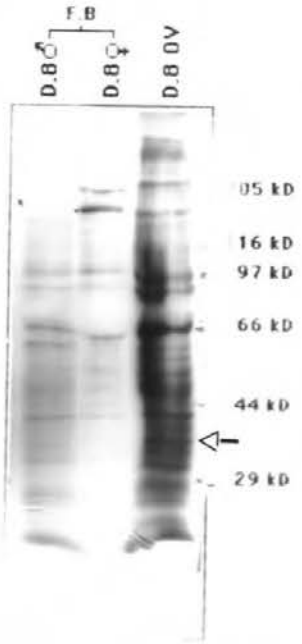
Panel b) Day 8 pharate adult , Lanes - 1) male F.B, 2) female F.B, and 3) Ovariole. High molecular weight protein markers from sigma were used.

FIGURE- 11 c Immunoprecipitation of male, female D8 fat body, vs ovariole ^{35}S proteins with *Galleria* YP4 anti sera.

To confirm the ovariole specificity of the 37 kDa, YP4 yolk polypeptide, same TCA precipitated counts (20,000 to 10,000 dpm) of ^{35}S -methionine labeled proteins from fat body and ovarioles were incubated with antisera raised against *Galleria* YP4 yolk polypeptide. Washed immuno-precipitates from each incubated samples were loaded on a 5-20% gradient polyacrylamide gel, as indicated below. The positions of prelabelled molecular weight markers (lanes 1 & 6) are shown on the right. In the autoradiogram, lanes from left to right show immuno-precipitate from 2) D6 3) D8 male fat body, 4) D5/6 female fat body, 5) D5 ovarioles 7) D8 female fat body and 8) D8 ovariole.



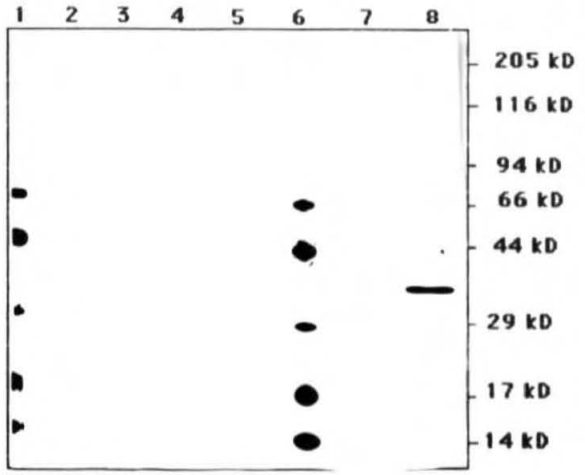
a



In vivo ^{35}S-Methionine labelled proteins

b

C



Immunoprecipitation Analysis of Fat body & ovariole proteins

with YP4- Antisera

F11 cDNA Codes for YP4

Since the cDNA appeared to have the entire reading frame including the start codon, to test whether F11 cDNA in fact encodes for the YP4 polypeptide, the N-terminal amino acid sequence of mature YP4 protein was determined and compared to the deduced amino acid sequence of F11 cDNA (fig.12). The first 10 amino acids of the purified YP4 protein are identical to the amino acid residues #18 to #28 of the deduced polypeptide (Fig.12a,underlined). The presence of a hydrophobic sequence at the N-terminal end of the deduced polypeptide (Fig.12 b) which apparently was cleaved off from the mature YP4 protein suggests that it is the signal peptide of this secreted protein. Thus, it was concluded that the F11 cDNA encodes the *Galleria* YP4.

The Single Copy YP4 Gene Is Intronless Within The Coding Region

The yolk protein genes in *Locusta* (Locke et al, 1987), mosquito (Romans, 1990) and in vertebrates appear to be multiple copy genes while those of *Drosophila* (Hung and Wensink, 1986), *B. mori* ESP (Sato and Yamashita, 1991) and *A. grandis* (Trewitt et al, 1991) appear to be single copy genes. Therefore, in order to determine the copy number of the YP4 gene represented in the *Galleria* genome, Southern analyses were done on genomic DNA from *Galleria* digested with restriction enzymes EcoR I and Pvu II (that do not cleave within the cDNA). The results suggest that the YP4 gene coding sequence is contained within a genomic region as small as 2.0 kb (fig.13A, B, lane 1) and also that yp4 gene exists as a single copy gene in the *Galleria* genome.

Another Southern blot analysis of genomic DNA restricted with EcoR I and combinations of Sma I, Acc I and PstI enzymes that cut within the F11 cDNA was also done to determine if introns were present within the yp4 gene. A blot of double digested electrophoresed genomic DNA (Fig. 13B) was hybridized to nicktranslated full length F11 cDNA insert. The results show that DNA digested with Acc I alone hybridizes to a ~850 bp and a ~600 bp fragments for a total of ~1.45 kb encompasing the YP4 encoding

Figure -12 N-terminal amino acid sequence of the *Galleria* yolk poly peptide-YP4 compared to the deduced amino acid sequence of the F11 cDNA (a) and its hydropathy plot (b)

a) Deduced amino acid sequence at the 5'end of the cDNA F11. The signal sequence cleavage site between Ala and Lys, is marked by an arrow.

b) Hydropathy plot was obtained using PEPPLOT program. The Y-axis, indicates hydrophobicity.

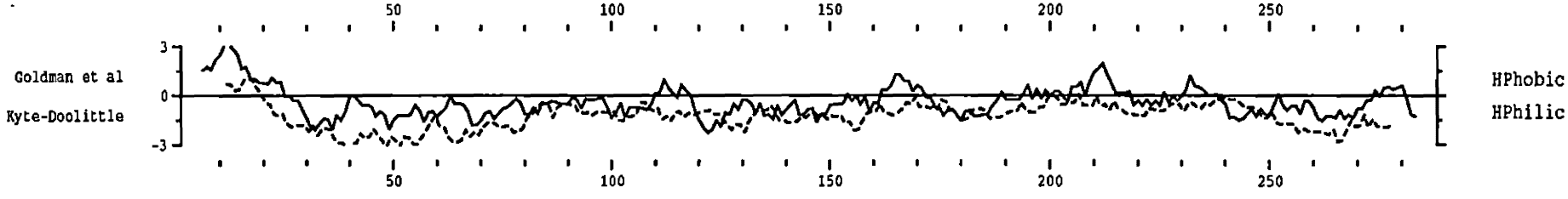
c) N-terminal sequence of the YP4 protein.

F 11 cDNA

5' AAAATGGTGTCGAAGTATCTGGCAATTTTGCTCTTCATCCCGATAATCTA
M V S K Y L A I L L F I P I I Y

CGCTAAAATCCAAGTGAATGTCGTCGCTTCTGAAAACGAGGCG.... -3'
A K I Q V N V V A L E N

HYDROPATHY PLOT



YOLK PROTEIN 4 [YP4] N-TERMINAL AMINO ACID SEQUENCE

1 2 3 4 5 6 7 8 9 10 11

LYS ILE GLN VAL ASN VAL VAL ALA LEU GLU ASN

K I Q V N V V A L E N

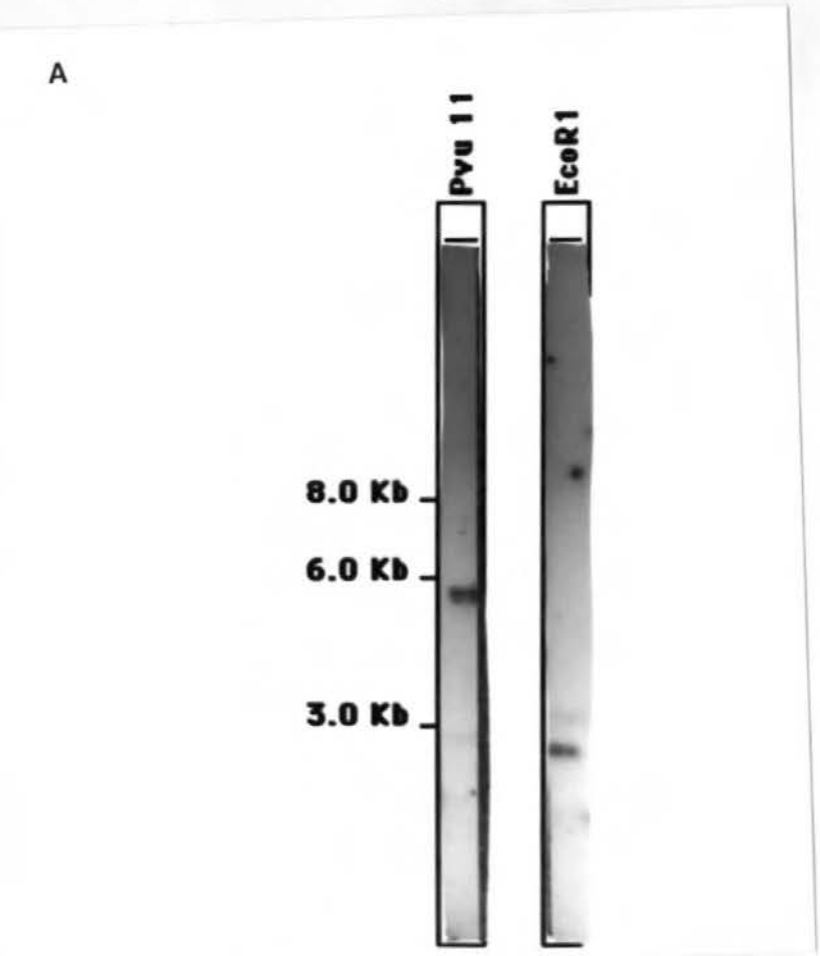
Figure -13 Genomic Southern analysis with F11 cDNA

High molecular weight genomic DNA was digested with the enzymes as indicated below was hybridized to nick translated YP4 cDNA (F11) . While the blot shown in a) was washed at 65°C and blot b) was washed at 62°C. The autoradiograms are shown in figures a) and b).

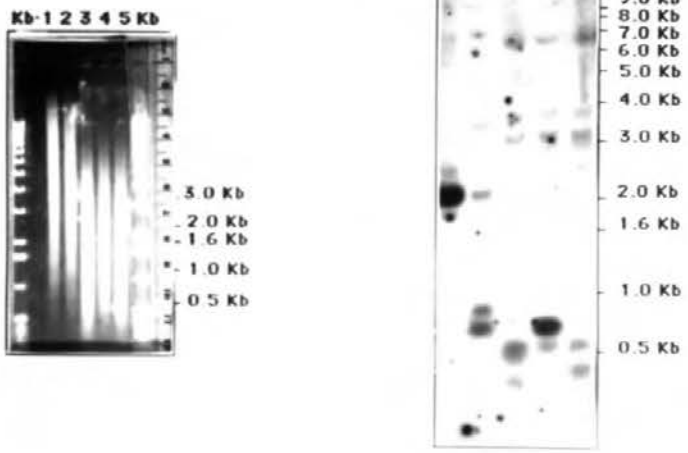
A) 10 ug/lane genomic DNA, digested extensively with either Pvu II or EcoR I were size fractionated on 0.6% agarose gel in Tris-acetate buffer (TAE).

B) 20ug/ lane DNA was digested extensively with the following restriction enzymes: lane 1= EcoR I, lane 2= EcoR I / Sma I, lane3 = Sma I / Acc I, lane 4 = Acc I, lane 5 = Acc I/ Pst I. The DNA was fractionated on a 0.7% agarose gel TAE and 0.5 ug/ml ethidium bromide and photographed under UV light (left panel).

As the autoradiogram of a blot washed under stringency conditions (65°C / 0.1x SSC) shows, EcoR I and Pvu II digestion produced single fragments of 2.0 and 5.5 kb respectively.


A
Pvu 1 1
EcoR1
8.0 Kb
6.0 Kb
3.0 Kb

B.


Kb 1 2 3 4 5 Kb
3.0 Kb
2.0 Kb
1.6 Kb
1.0 Kb
0.5 Kb
1 2 3 4 5
9.0 Kb
8.0 Kb
7.0 Kb
6.0 Kb
5.0 Kb
4.0 Kb
3.0 Kb
2.0 Kb
1.6 Kb
1.0 Kb
0.5 Kb

sequences (figure 13B, right panel). The larger intensely hybridizing Acc I fragment (figure.13B, left panel, lane 4) is cut by either Sma I (lane 3) or Pst I (lane 5) generating ~500 bp and ~300bp (lane 5), and two ~450-400bp (lane 3). Based on the cDNA restriction map, the larger Acc I fragment contains the YP4 coding region 3' of the Acc I site. As expected, the Acc I /Sma I (lane 3) and Acc I / Pst I (lane 5) produced fragments close to the expected 558bp and 452bp, suggesting that the YP4 gene is intronless in these regions. The faintly hybridizing higher molecular weight bands in the 3.0, 4.0, 6.5 kb region appear to be due to partial digestion.

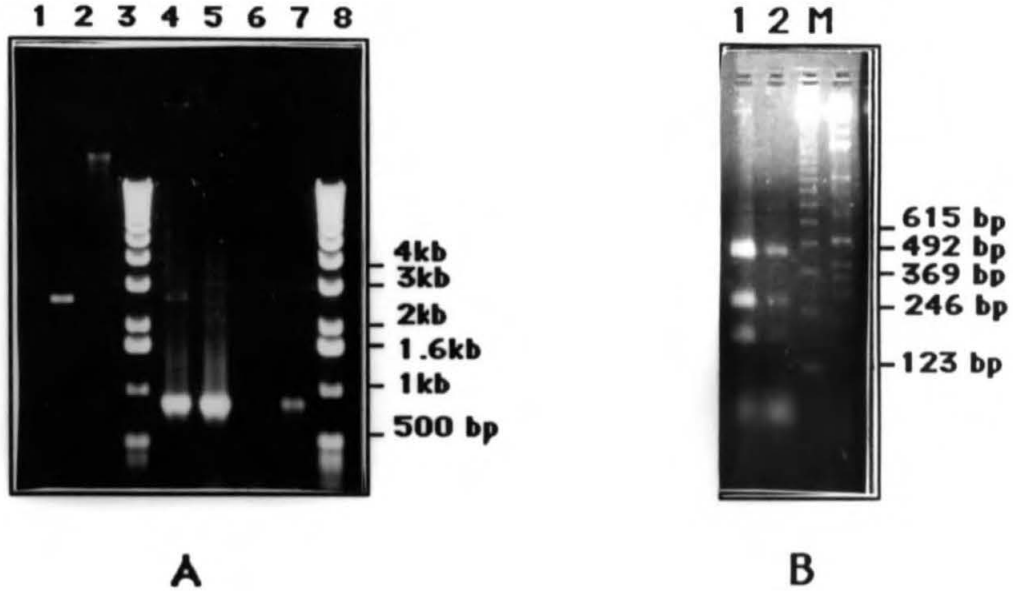
Southern analysis indicated that the YP4 coding sequences are contained within a maximum of 2.0 kb EcoR I and possibly in a 1.45 kb Acc I genomic fragment, I chose to amplify a YP4-encoding genomic fragment by PCR to verify the absence of introns. Genomic DNA and F11 cDNA were used as templates (Fig.14a, lanes 1 and 2) in separate PCR reactions to amplify the DNA sequences between the primers, very close to the 5' (+6 to +24) and 3'(+878 to +850) ends of the F11 cDNA . Figure.14A shows the photograph of the 0.7% agarose gel (0.5% ethidium bromide) in which the cDNA and genomic PCR amplified products were electrophoresed. The PCR products from both cDNA (fig.14A lane 4 and 5) and genomic DNA (lane 7) were 870 bp in size, the length expected for the cDNA PCR product. In order to confirm that there is no small size introns (50-20 bp) that may not have been detected, the PCR products were digested with Pst I and Acc I, two enzymes that have recognition sites within the coding region and size fractionated in 2.5% agarose gel for better resolution. As figure 14B, lanes 1 and 2 show, both the genomic and cDNAs gave same size fragments (452, 252 and 170 bp). The absence of any difference in the sizes between the genomic and cDNA fragments, at this resolution clearly demonstrates that the *Galleria* YP4 gene is intronless, at least within the coding region between the primers used.

Figure 14: PCR analysis of genomic vs F11 cDNA

Using an 18-mer (+6 to +23 bp), 5' -GGTGTCGAAGTATCTGGC- 3' as the forward primer and another 18-mer (+938bp to +920bp), 5' -GTACATTGTTCTCAGC-3' as the reverse primer, genomic DNA and cDNA were PCR amplified (90°C/1', 60°C/1', 72°C/3') using Taq polymarase.

a) The left panel shows the gel photographed under UV light. Lane 1 = cDNA template, lane2 = Genomic DNA template, lane 4 and 5 = PCR products from cDNA templates, lane 6 = control reaction in which no template was added, lane 7 = PCR products from genomic DNA. Lanes 3 and 8 = 1kb molecular weight marker.

b) The right panel shows the PCR products digested with restriction enzymes Acc I and PstI. Lane 1 = from the cDNA, lane 2 = from the genomic DNA and lane 3 = 123 bp ladder (BRL). In both gels, the faint band showing up at the gel front of each PCR reaction lanes are the excess primers.


1 2 3 4 5 6 7 8
4kb
3kb
2kb
1.6kb
1kb
500 bp
A
1 2 M
615 bp
492 bp
369 bp
246 bp
123 bp
B

F20 (0v 5) cDNA has a single open reading frame

Restriction analysis of F20 (Ov 5) cDNA (figure 15) showed the presence of two Sty 1 and single Cla1, SstI, Sst II, Sma I, ScaI and Acc I restriction sites. Digesting with EcoR I and Cla1, released two fragments, a 345 bp and a 1269 bp fragments (fig.15a,b) which were (Klenow filled) subcloned into Hinc II site of plasmid pGEM-3Z. A 415 bp internal Sty I fragment of F20 was also (Klenow filled) subcloned into the Hinc II site of pGEM-3Z. These subclones containing inserts of 345 bp, 1269 bp and 415 bp sizes were designated F-E/C, F-C/E and F-Sty. The subclone Fs was generated by religating the same vector containing the 5', 765 bp Sma I fragment. All these subclones were sequenced from both directions using the SP6 and T7 universal primers. To sequence across restriction site junctions and internal portions beyond the resolution of the sequencing gel, oligonucleotide primers were also employed. As sequence information became available, specific oligonucleotide primers P1 (332- 351bp), and F2 (983 -966 bp) were used to enable sequencing the internal regions. The sequencing strategy is shown in fig.15b.

The entire 1.6 kb F20 cDNA was sequenced in an effort to identify the polypeptide coded for, by the F20 cDNA and the sequence is shown in figure 16. The sequence information was also confirmed with the restriction enzyme maps prepared by gel analyses of single and double enzyme digestions of the plasmid clone (15 a). In addition, a cross hybridizing clone (designated 1K)with a 1kb insert, was also sequenced partially and was found to start from 517 bp of F20 cDNA.

The nucleotides are numbered +1 from the 5'end of the F20 cDNA. It has a single open reading frame (fig.16) of 1512 bp and a TAA termination codon (at 1515bp) at the 3' end. The consensus eukaryotic polyadenylation signal sequence AATAAA was not found following the termination codon. However, an AT rich sequence ATATAATATTAT which might serve as a potential poly A+ addition signal as in bicoid (Berleth et al.,1988), cyclin (Lehner and O' Farrell, 1989) and Orb (Valerie Lantz et al,1992) messages of *Drosophila*, was found 40 bp downstream of the first termination codon there by

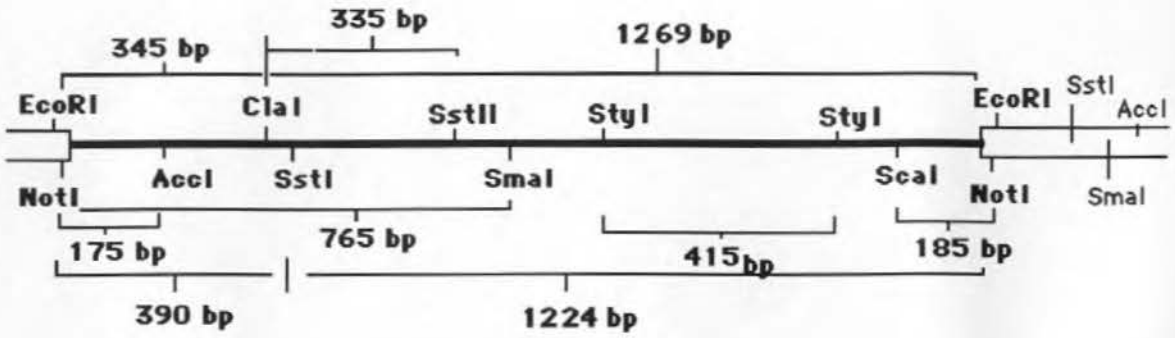
Figure 15a: Restriction analysis of F20 cDNA

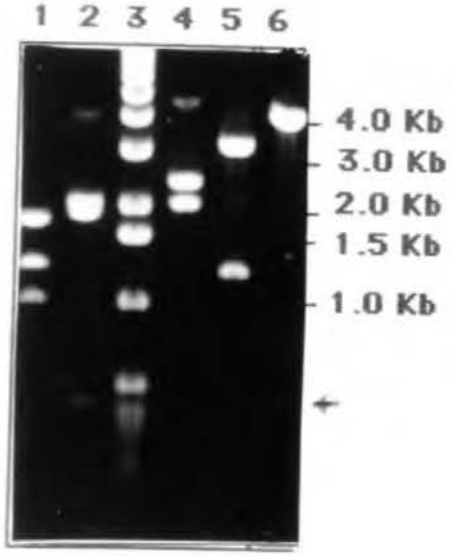
F20 subclone (in pGEM-3Z) was digested with endonucleases and the products were analyzed by 0.7% agarose gel electrophoresis in Tris-Acetate buffer. GEL1: Lane 1- ScaI / SstI, lane 2- ScaI/Sty I, lane 4 -ScaI, lane 5- SstI, lane 6- StyI digested F20 subclone DNA.. Lane 3- 1 Kb DNA ladder as molecular weight marker.

GEL2: Lane 1- Acc I, Lane 2- Cla I, Lane 3- Acc I / Sst II, Lane 4- Cla I / Acc I.

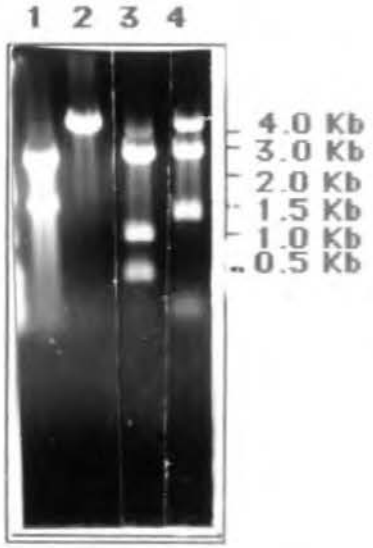
A map of F20 cDNA is given above the gel figures.

MAP OF F20 cDNA





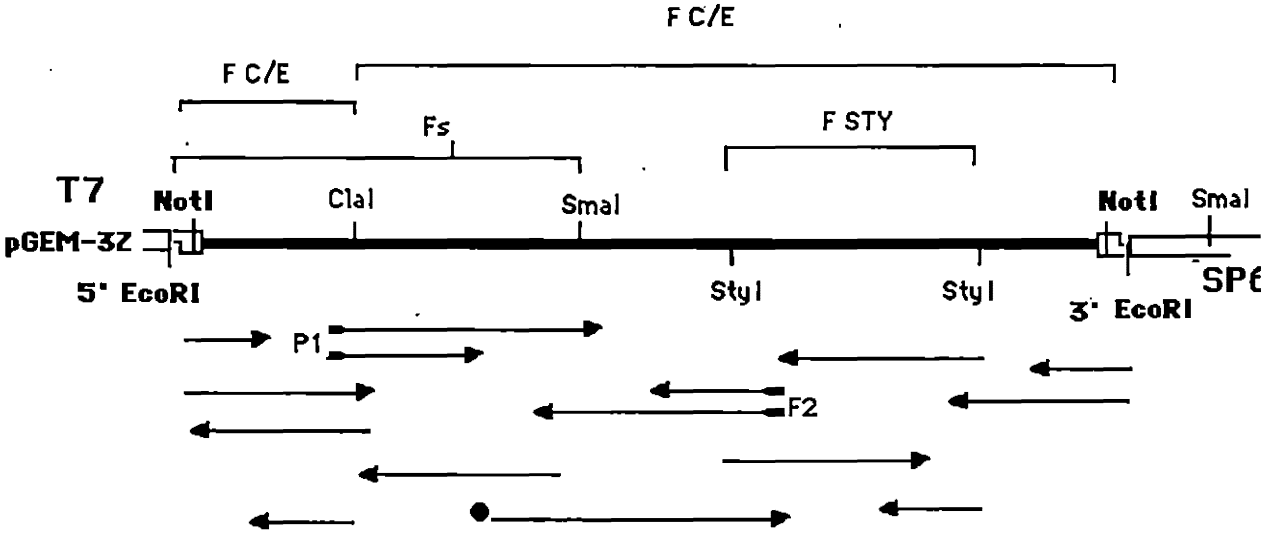
A



B

FIGURE -15b. Sequencing strategy of F20 cDNA.

The strategy consisted in sequencing the termini of each subclone using vector sequence primers. The symbols of restriction enzyme sites are as marked on figure-15a. Each arrow indicates the directions of sequencing and the extent of sequence data obtained from at least two sequencing trials. New subclones FE/C (345bp), FC/E (1275bp), FSl (765bp) and F Sty (415bp) generated and sequenced in both directions using the T7 and SP6 primers. P1 (332 bp to 351bp) and F2, (983 bp to 966bp) are two internal oligonucleotide primers that were also used for sequencing. Another independent cross hybridizing cDNA clone designated 1K with an insert of 1.0 kb (from 517bp of F20) was also partially sequenced.



F20 Sequencing Strategy

suggesting that the F20 may contain the 3' end of the coding region. Such sequence was not reported in *Galleria* gene sequences known so far.

Since the start codon was not found and the putative 5' end was missing from the 1.6 kb F20 cDNA, the coding strand of the F20 cDNA clone was further confirmed by probing northern blot with *in vitro* transcribed ^{32}P-CTP labeled sense or antisense RNA. Linearized F20 cDNA in pGEM-3Z was used to generate the labeled probe using either T7 or SP6 RNA polymerase. The SP6 RNA polymerase transcribed the anti-sense RNA probe, that hybridized to the 2.0 kb vitellogenic F20 transcripts on the northern blot (fig.17).

The open reading frame of F20 sequence (fig.16) indicate that it codes for a functional transcript that can potentially encode a 504 aa polypeptide with calculated molecular weight of 58 kDa. The fact that this mRNA is present only in follicle cells and is expressed only during vitellogenic stages suggest that F20 codes for a polypeptide with the same size, tissue and developmental stage-specific expression as of YP2 yolk polypeptide (74 kDa).

F20 GENE: Copy Number and Structure

In order to determine the F20 copy number, a genomic Southern analysis was done with EcoR I and Pvu II digested *Galleria* genomic DNA the enzymes that do not cleave the F20 cDNA. Southern blots were probed with nick translated 1.6 kb F20 cDNA. The autoradiogram of this blot is shown in figure 18a. Three Pvu II bands (12kb, 4kb and 2kb) and three EcoR I bands (9 kb, 6.5 kb and 3.2 kb) hybridized to the probe. The genomic EcoR I fragments totaling about ~16.7 kb DNA (fig.18a), indicate that the F20 hybridizing sequences occupy a maximum genomic mass of ~16.7 kb DNA . The 5.0 kb EcoR I band was weakly hybridizing already at 65^{0}C. As shown in fig.18b when the washes were done at 68^{0}C, the 3.2 band was less intense than in 18a. The EcoR I produced, 9.0 and 6.5 kb fragments showed equally strong signal (fig.18b.lane 1)

FIGURE 16: Nucleotide and deduced amino acid sequence of F20 cDNA.

The nucleotides are numbered (given on the left side) from the very 5' end of the cDNA, starting with + 1 although it is not the true 5' end of the coding frame. The potential polyadenylation signal at 1554bp is underlined and the stop codon TAA at 1515bp, is identified with an asterisk. The deduced amino acid sequence is numbered on the right side. The four N-glycosylation sites are in bold letters and the serine rich region (for potential phosphorylation) of the polypeptide, are identified by italics. The potential poly A+ signal sequence is under lined.

F20 cDNA and deduced amino acid sequence

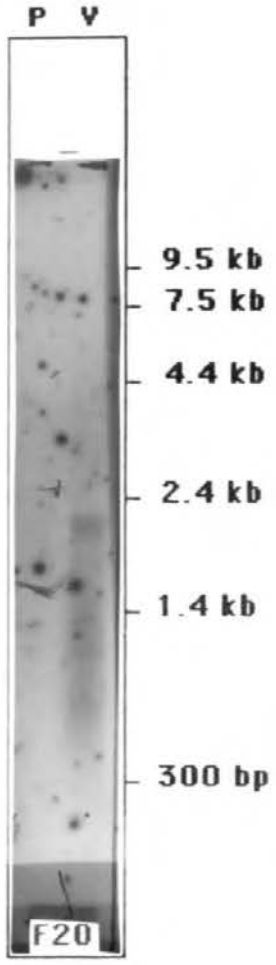
```
    CCGGAGAGTCTCTGCAATACCTGGACGGATACTACCAACAGCATCCTCGTGTACAGAGGG
  1 ---------+---------+---------+---------+---------+---------+
     G  E  S  L  Q  Y  L  N  G  Y  Y  Q  Q  H  P  N  V  Q  R  E 20

    AGTACAGCAAGGTCCACAGTTCCGAATATGACGAAACACTACAAACAAGCAAACGCTCTT
 61 ---------+---------+---------+---------+---------+---------+
     Y  S  K  V  H  S  S  E  Y  D  E  T  L  Q  T  S  K  R  S  S 40

    CATCAAGCGAATCATCCGGGGAACACCGTCCCGAGGACATGTACATGCCTCCCGTCTACG
121 ---------+---------+---------+---------+---------+---------+
     S  S  E  S  S  G  E  H  R  P  E  D  M  Y  M  P  P  V  Y  A 60

    CCGACGAGAATATGACTGCTCAAATGATGAAGAAACCTGTAGAAGCACCGCACGGTGACG
181 ---------+---------+---------+---------+---------+---------+
     D  E  N  M  T  A  Q  M  M  K  K  P  V  E  A  P  H  G  D  A 80

    CTATCAGTTCCAGGGCATCAAAATCCCTCGGCCCCCACTCGCAGATCAAGAAGAAGGAAC
241 ---------+---------+---------+---------+---------+---------+
     I  S  S  R  A  S  K  S  L  G  P  H  S  Q  I  K  K  K  E  Q 100

    AAATCGAAAAACTCTTCGCCGACGCACAGATGTCAATGAAACACATCGATGAAGACAGCC
301 ---------+---------+---------+---------+---------+---------+
     I  E  K  L  F  A  D  A  Q  M  S  M  K  H  I  D  E  D  S  Q 120

    AAAAGACATTCCACAAAATGTACGAGCTCGCCGTCCAGAAGGAAGACGAGGACGTTTATT
361 ---------+---------+---------+---------+---------+---------+
     K  T  F  H  K  M  Y  E  L  A  V  Q  K  E  D  E  D  V  Y  F 140

    TTAACGCTACCCAACTATTGAAGAAATATGGGTATCCAGTAGAGGAGCACACCATACAGA
421 ---------+---------+---------+---------+---------+---------+
     N  A  T  Q  L  L  K  K  Y  G  Y  P  V  E  E  H  T  I  Q  T 160

    CTGGCGATGGCTACTACCTCACGGTGTTCCGCATCATGAAATACACGGCCAGGCGTACTC
481 ---------+---------+---------+---------+---------+---------+
     G  D  G  Y  Y  L  T  V  F  R  I  M  K  Y  T  A  R  R  T  P 180
```

```
    CGAGCGTGGCATCGAAGGGCGTCGTCCTACTAATGCACGGCCTATACGGCAGCGCACGAC
541 ---------+---------+---------+---------+---------+---------+

    S  V  A  S  K  G  V  V  L  L  M  H  G  L  Y  G  S  A  R  L 200

    TGGCTCCTCATGGGCCCCGAATACTCCTAGCTTACTTGTTAGCTGACGAAGGCTACGAAG
601 ---------+---------+---------+---------+---------+---------+

    A  P  H  G  P  R  I  L  L  A  Y  L  L  A  D  E  G  Y  E  V 220

   TATGGCTAGGCAACGTCCGCGGAAACAAATATGGCCGCCAACACGTCTCGAAACACCCAGC
661 ---------+---------+---------+---------+---------+---------+

    W  L  G  N  V  R  G  N  K  Y  G  G  Q  H  V  S  K  H  P  A 240

    TCAGAAGGACTTCTGGCAATTCAGGGTTGATGAGATCGCCCGGGTTGATTTGCCATCCC
721 ---------+---------+---------+---------+---------+---------+

    Q  K  D  F  W  Q  F  R  V  D  E  I  A  R  V  D  L  P  S  L 260

    TGATCGACTACGTCCTGCAAATCACTGGGCAAAAGAAGTTGTACTACGTTGGTTATGACC
781 ---------+---------+---------+---------+---------+---------+

    I  D  Y  V  L  Q  I  T  G  Q  K  K  L  Y  Y  V  G  Y  D  Q 280

    AGGGTACCACCGCATTCTTGCCTATGGCGTCCACTATGCCCGAATACGGCGACAAAATAA
841 ---------+---------+---------+---------+---------+---------+

    G  T  T  A  F  L  P  M  A  S  T  M  P  E  Y  G  D  K  I  I 300

    TAAAGATGTACGCTATGGCCCCAATGGTGTACATGAGTTCCATGGTCCGCATGATTGCTC
901 ---------+---------+---------+---------+---------+---------+

    K  M  Y  A  M  A  P  M  V  Y  M  S  S  M  V  R  M  I  A  P 320

    CGACCAGCGATCTCCATGAGCAACTCAGCCCATACTTTATGGATGGCGAGTTCCACCCGT
961 ---------+---------+---------+---------+---------+---------+

    T  S  D  L  H  E  Q  L  S  P  Y  F  M  D  G  E  F  H  P  S 340

    CCAAAGAACTGCTTAAAACATTGGGAGGCGAAATGTGCGAAAAGGAGATCGGATGTAGGA
1021 ---------+---------+---------+---------+---------+---------+

    K  E  L  L  K  T  L  G  G  E  M  C  E  K  E  I  G  C  R  K 360
```

```
     AGATTTGCTCCAACCTCAACTTTGTCATGTCCGGAGTAAATTTGGAGCACATGGAACCCG
1081 ---------+---------+---------+---------+---------+---------+

     I  C  S  N  L  N  F  V  M  S  G  V  N  L  E  H  M  E  P  A 380

     CTCAGATTCCGATGATCACAGGGCACTTGCGCTGGAGGTTCGACCAGACAAATAAAGCAG
1141 ---------+---------+---------+---------+---------+---------+

     Q  I  P  M  I  T  G  H  L  R  W  R  F  D  Q  T  N  K  A  V 400

     TACAGCCAATCGTTGCTTCCCGTGAATTTAGAATGTACGATCATGGCGCTAAGATCAACA
1201 ---------+---------+---------+---------+---------+---------+

     Q  P  I  V  A  S  R  E  F  R  M  Y  D  H  G  A  K  I  N  K 420

     AGAAGATGTATGGTTCAGTTCAGCCACCAGTATATGATGTATCCAAGATCCAGACCCCTG
1261 ---------+---------+---------+---------+---------+---------+

     K  M  Y  G  S  V  Q  P  P  V  Y  D  V  S  K  I  Q  T  P  V 440

     TCGTTTTGTACTACAGTGAGGAGGATTGGTTGTCGCACCCCAAGGACGTCGAGAGGCTGC
1321 ---------+---------+---------+---------+---------+---------+

     V  L  Y  Y  S  E  E  D  W  L  S  H  P  K  D  V  E  R  L  H 460

     ACAGGGAACTGCCCAATGTGACGGAGTACTACAAAGTGCCAGAGGGGTACTTCGCTCACA
1381 ---------+---------+---------+---------+---------+---------+

     R  E  L  P  N  V  T  E  Y  Y  K  V  P  E  G  Y  F  A  H  M 480

     TGGATTACCAGCATTACAAGAAGGCACCTGAAATGGTGTACACGAGGCTGATCAAATCTA
1441 ---------+---------+---------+---------+---------+---------+

     D  Y  Q  H  Y  K  K  A  P  E  M  V  Y  T  R  L  I  K  S  M 500

     TGAATAGCTCCTCTTAAGTATTGTATTAGTGTGTGAAACCTTTTATATATAATATTATCA
1501 ---------+---------+---------+---------+---------+---------+

     N  S  S  S  *  V  L  Y  *  C  V  K  P  F  I  Y  N  I  I  M -

     TGTAGGTGTGTTATTAATTATGTCTAACTAACTGTAACTTTATTGAAGCATAAT
1561 ---------+---------+---------+---------+---------+-------
      *  V  C  Y  *  L  C  L  T  N  C  N  F  I  E  A  *  C  G   -
```

FIGURE -17. The coding strand was detected by Northern analyses with single stranded RNA probes.

Northern blot of poly A+ RNA from an equivalent of five 1) pre-vitellogenic and 2) vitellogenic stage follicles was hybridized to ^{32}P-CTP labeled RNA probes, transcribed in vitro with SP6 RNA-Polymerase from linearized F20 (Cla I digested) template. RNA ladder (BRL) was used as molecular weight marker and the sizes are given by the side of the blot.


P V
9.5 kb
7.5 kb
4.4 kb
2.4 kb
1.4 kb
300 bp
F20

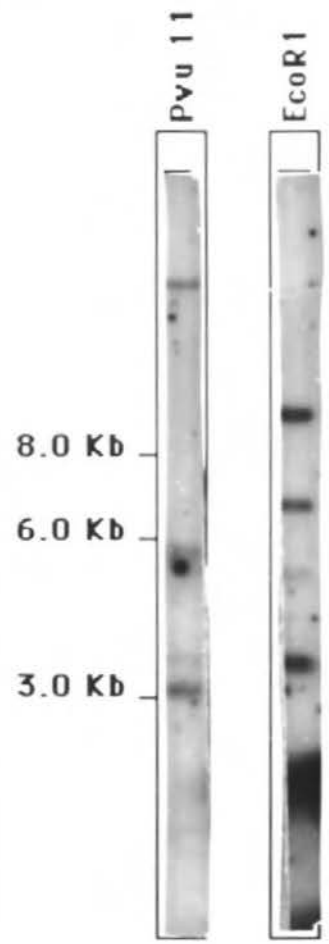
even at this highly stringent wash. Since there is no EcoR I site within the F20 cDNA, the presence of two equally intense bands and the weak bands even at stringent conditions indicate that F20 gene is either a single copy gene with introns or belongs to a small gene family. Southern analysis was also done with genomic DNA digested with Cla I, and Sst II, enzymes that have single recognition sites within the F20 cDNA either singly or together with EcoR I. The data is shown in figure 18b. Sst II along with EcoR I (fig.18b, lane 3) produce four intense bands (8.0, 4.5, 2.9, 1.1 kb) and a weak band (2.3 kb). A weak band of slightly larger size (~ 2.7 kb) is also produced with EcoR I single digestion (fig.18b, lane 1). As lane2 shows, four fragments of ~3kb, 1kb, 0.75 kb and 0.35bp are produced with Cla I/Sst II double digestion. While the ~350 bp fragment is the expected Cla I / Sst II fragment size (from cDNA map) presence of at least five fragments (3.1, 2.2, 1.0, 0.75 and 0.35 kb size), suggest the presence of introns. Single enzyme digestion with Cla I (fig.18b. lane 4), show three intense and two weaker bands hybridizing to the probe, at least three more fragments than expected for enzymes recognizing only single sites within the cDNA .

The weakly hybridizing bands on the Southern blot may result from either partial identity between probe and target sequence, or from dilution of the specific target in a given band by large intron sequences. When the cDNA restriction fragment sizes / patterns (fig15a) are compared with the fragment number and their sizes seen in Southern blots (fig.18b), the total amounts of genomic material that hybridizes to F20 cDNA is greater than that would be expected of a single copy intronless gene. Therefore it is reasonable to suggest that the gene encoding F20, either has introns and/or belong to a small gene family.

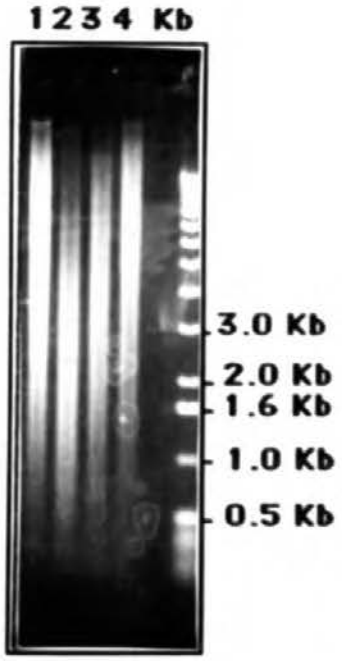
Figure 18: Genomic Southern analysis with F20 cDNA indicate that it represents a small gene family with introns.

Autoradiograms of Southern blots with a) 10 ug/lane genomic DNA, digested with Pvu II or EcoR I were size fractionated on 0.6% agarose gel in TAE buffer.
b) Southern blots of DNA digested and fractionated / lane) on a 0.7% agarose gel in TAE as left panel shows lane 1= EcoR I, lane 2= Sst II / Cla I, lane3 = EcoR I / Sst II, lane 4 = Cla I. Membranes was hybridized to nick translated 1.6 kb F20 insert and washes were done at 65°C (panel A) and 68°C (panel B). The blot was exposed to X-ray film for 5 days at -70°C with intensifying screen.

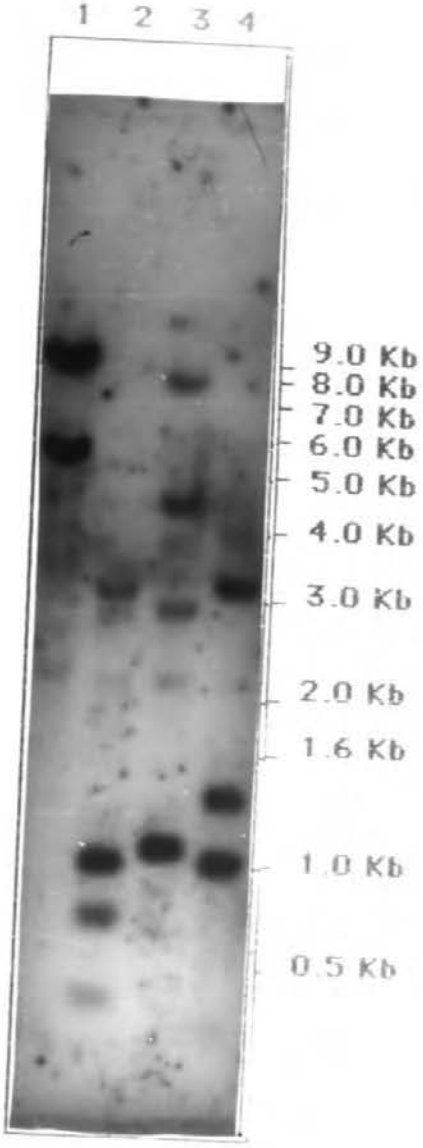
A



F 20


1 2 3 4 Kb
3.0 Kb
2.0 Kb
1.6 Kb
1.0 Kb
0.5 Kb

B


1 2 3 4
9.0 Kb
8.0 Kb
7.0 Kb
6.0 Kb
5.0 Kb
4.0 Kb
3.0 Kb
2.0 Kb
1.6 Kb
1.0 Kb
0.5 Kb

Yolk polypeptide-YP4 Shows Similarity to Spherulin-2A

Comparison of the YP4 to the protein sequences in the data bank SWISSPROT* using the FASTA program showed similarity to slime mold spherulin-2A with 35.2% identity in a region of 213 amino acids (fig.19, i). When considering the conservative amino acid substitutions (118), the similarity is 74%. The similarity is significant and higher than if it would be random or analyzed by "shuffled" YP4 amino acid sequence still maintaining the amino acid composition. The conserved positions of the two adjacent N-glycosylation sites and four proline residues may indicate some structural and/or functional similarities. Spherulin-2a, is an encystment stage specific glycosylated, protein from the slime mold *Physarum polycephallum.* (Bernier et al, 1987). Since it lacks a signal sequence, spherulin-2a is believed to remain inside the unicellular structure.

Comparison of YP4 deduced sequence to NIH data bank also indicated that YP4 has small regions of similarity to metalloproteinase and thrombomodulin also known as fetomodulin (Dittman et al, 1988). The similarity to bovine interstitial collaginase precursor (matrix metalloproteinase) falls in two short regions from aa 84 to 104 and aa 33 to 66 showing 28% and 26% identities. When the conservative amino acid replacements are considered the similarity increases to 76% and 52% respectively. Similarly, two regions from aa 24 to 49 and aa 176 to 202, showed 30% and 37% identities to thrombomodulin. The similarity level increased to 53% and 62% when the conservative amino acid changes are considered.

Figure 19 : Deduced amino acid sequence comparison

Amino acid sequence alignments obtained by FASTA program of Genetics Computer Group (GCG) are shown. The vertical bars indicate identities while colons show the conservative replacements.

i) Homology of YP4 protein with

Sperulin-2A an encystment protein from Physarum polycephallum.

In a 213 bp region, there are 75 identities (35.2%) and 118 conservative replacements adding up for a homology of 74%. The position of two adjacent glycosylation sites and position of four proline residues are also conserved.

The shuffled YP4 sequence, still maintaining the amino acid composition gave only shorter regions with lower homologies to random sequences in the data bank.

YP4 / Spherulin-2a similarity (35.2% identity in 213 aa overlap)

```
            20        30        40        50        60        70
O118.P IPIIYAKIQVNVVASENEAETGEPGWKNVDIITDNERYTFQLTDNNLKNAVQSYFGQRPD
                                    |:||::| :| |:::|| :|||:: |::|:
Sr2a_P          MAFQVNAHVGNRTASSHGVVERIMTDSDRRAFGLDGDNLFRAVERFRGRWPT
                        10        20        30        40        50

            80              90       100       110       120
O118.P DAFLRSPTPWG--DLYQVY---GWPQVARSLSSSESKYLQVSSKPSIILTQHFRNNSTQP
       :|::|||:  |  ||||:|   ||:||: :|:: :|:  : ::::::::|::::|||: |
Sr2a_P GAWVRSPAIAGGVDLYQAYAHQGWRQVVTRLEPISSTIHHPNTDRTTVVTARLSNNSSFP
            60        70        80        90       100       110

       130       140       150       160       170       180
O118.P ATFKAQIQQQVQNTVTSTWEKGGELTVGQEIEYGFDIKVVSVGGKTSFSYTSRWGESVSK
       ::| |:: ::: |::|::|::: :::|||:::|:::: |:|:|||:|: :|| : ::
Sr2a_P GEFFANLSNETTNSATTSWSSTHGIEVGQSVSYSIGV----VSGETSFGYSYQWGRGGEQ
            120       130       140       150       160

       190       200       210       220       230       240
O118.P SETVTVGSESGVEITLEPGQAIIAELLATRGTMEIQVDYEATLSGATAVNYANTFKGHHH
       ::: :|:  :||:: |:|||::|: |||::|  | : | |:|:| :| |:: : :| ||
Sr2a_P TTASSVSFVTGVTVHLQPGQGVIVRLLAEQGWARITTRYRASLTGHVAQNFNPPHQG-HH
      170       180       190       200       210       220

       250       260       270       280       290       300
O118.P FWASGHQCCDDVWRLEPHRSLPGSYQARLLLQLSRCDQRCX
       |||
Sr2a_P FWAHSVNSILQASGLPTQIFIENTVDVGFFANSHVDMQDLVTGVIVPIGTDKIFRPLALK
       230       240       250       260       270       280
```

F20 encoded vitellogenic ovariole polypeptide shows similarity to *B. mori* ESP and mammalian triacylglycerole lipases

The deduced F20 polypeptide sequence was compared to amino acid sequences in the gene bank using the FASTA program revealing 42% identity to ESP of *B. mori* in a region of 383 amino acids and 33 to 30% identity in a region of 370 amino acids to human and rat triacylglyceriole lipases. When the conserved amino acids are considered the similarity is raised to 74%. The F20 and ESP show significant similarity in regions that are conserved between the rat and human triacylglycerol lipases. *Drosophila* YPs also show similar level of homology to other members in the lipase gene family. A lipid binding domain conserved among the mammalian lipases is shown in figure 19, ii b). In this lipid binding region *Drosophila* YPs show 50% identity to lipoprotein lipase over an 18 amino acid region. YP1 sequence comparison in this 18 amino acid region is shown in the figure b). *Galleria* F20 shows 68 % identity to human triacylglycerol lipase in the lipid binding region extending to 16 amino acids (19, ii b). *B.mori* ESP which also has the lipid binding domain has the conserved serine residue unmodified (19, iib). This serine residue is replaced by an aspartic acid in the F20 sequence while it is replaced by an asparagine in the *Drosophila* YP1. The significance of this observations is discussed later in discussion.

Regulation of oogenesis

Ovary Transplants in males

A method to identify the sex of last instar larvae

Ovary development and vitellogenesis in moths occur spontaneously in the pharate adult stage. Using the specific probes developed in this study to detect the expression of follicle specific genes YP4 and F20, we tested whether these ovary specific genes can be expressed normally in ovaries transplanted into males (in male milieu) or if female specific humoral factors are needed.

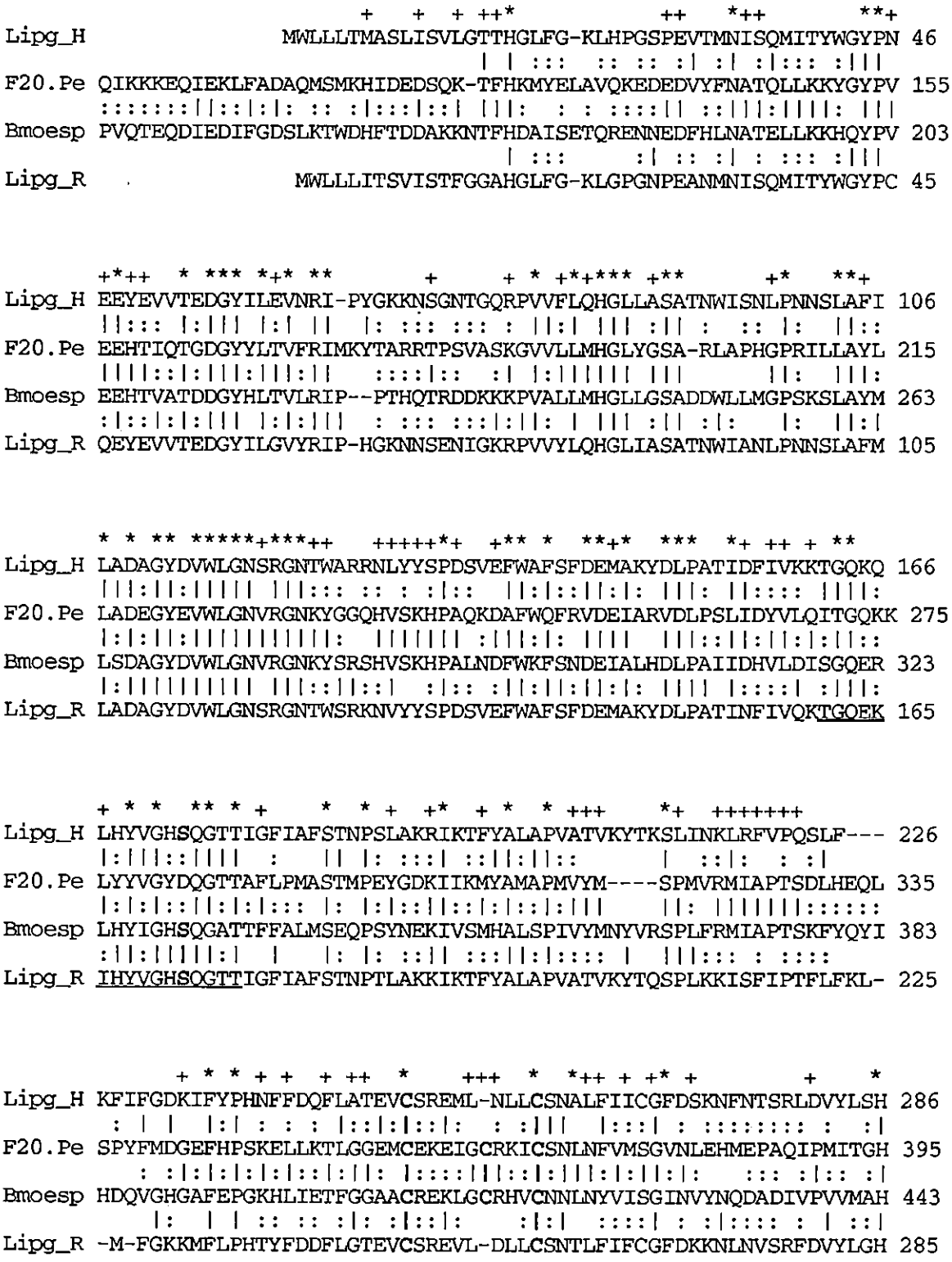
Figure 19 ii) Homology of F20 deduced amino acid sequence to *B. mori* ESP and mammalian triacylglycerol lipase

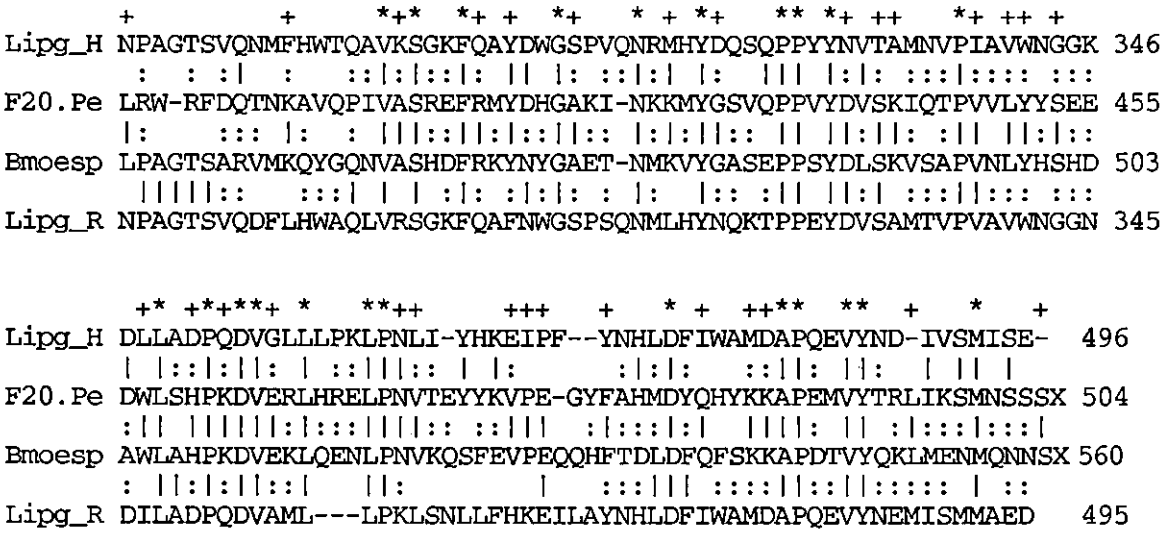
a) In a region of 383 amino acids, the deduced amino acid sequence of F20 shows 42% identity to Bombyx ESP and in a region of 370 amino acids, 33 to 30% identity to rat and human triacylglycerol lipases. The homology is particularly high around the lipase binding domain conserved in the mammalian lipases. The amino acid residues conserved between *B. mori* ESP and *Galleria* F20 are marked by "+" and those conserved between both lipases and the follicle specific proteins are marked by asterisks "*" above the sequence alignment. The conserved cysteine residues and the serine residue of lipid binding domain are bold lettered.

b) F20 shows homology to conserved lipid binding domain of mammalian lipases

The figure shows the conserved lipid binding domain from different mammalian lipases and insect follicle specific yolk proteins. The lipase binding domain in *Drosophila* YP1 showing high homology to lipoprotein lipase is also shown. The conserved active site serine residue, and the corresponding replacements in *Drosophila* YP1 and *Galleria* F20, is underlined.

Homology of F20 to B. mori ESP and mammalian lipases




Lipg_H NPAGTSVQNMFHWTQAVKSGKFQAYDWGSPVQNRMHYDQSQPPYYNVTAMNVPIAVWNGGK 346
F20.Pe LRW-RFDQTNKAVQPIVASREFRMYDHGAKI-NKKMYGSVQPPVYDVSKIQTPVVLYYSEE 455
Bmoesp LPAGTSARVMKQYGQNVASHDFRKYNYGAET-NMKVYGASEPPSYDLSKVSAPVNLYHSHD 503
Lipg_R NPAGTSVQDFLHWAQLVRSGKFQAFNWGSPSQNMLHYNQKTPPEYDVSAMTVPVAVWNGGN 345
Lipg_H DLLADPQDVGLLLPKLPNLI-YHKEIPF--YNHLDFIWAMDAPQEVYND-IVSMISE- 496
F20.Pe DWLSHPKDVERLHRELPNVTEYYKVPE-GYFAHMDYQHYKKAPEMVYTRLIKSMNSSSX 504
Bmoesp AWLAHPKDVEKLQENLPNVKQSFEVPEQQHFTDLDFQFSKKAPDTVYQKLMENMQNNSX 560
Lipg_R DILADPQDVAML---LPKLSNLLFHKEILAYNHLDFIWAMDAPQEVYNEMISMMAED 495

LIPID BINDING DOMAINS OF MAMMALIAN LIPASES AND INSECT FOLLICLE SPECIFIC YOLK PROTEINS

Human LCAT (Lecithin cholesterol Acetyl transferase)	173	Pro Val Phe Leu Ile GLY His Ser Leu Gly
Porcein pancreatic lipase	145	Asn Val His Val Ile Gly His Ser Leu Gly
Rat hepatic Lipase 1	140	Lys Val His Leu Ile Gly Tyr Ser Leu Gly
Rat hepatic Lipase 2	261	Ser Val His Leu Phe Ile Asp Ser Leu Gln
Bovin lipoprotein Lipase		Ser Val His Leu Phe Ile Asp Ser Leu Leu
Lipoprotein Lipase	124	Asp Asn Val His Leu Leu Gly Tyr Ser Leu Gly Ala
Drosophila YP1	247	Asp Thr Ile His Leu Ile Gly Gln Asn Val Gly
Bombyx ESP	317	Ser Gly Gln Glu Arg Leu His Tyr Ile Gly His Ser Gln Gly Ala Thr
***Galleria* F20**	272	Thr Gly Gln Lys Lys Leu Tyr Tyr Val Gly Tyr Asp Gln Gly Thr Thr
Rat lingual lipase	160	Thr Gly Gln Glu Lys Ile His Tyr Val Gly His Ser Gln Gly Thr Thr
Human Gastric Triacylglycerol Lipase	161	Thr Gly Gln Lys Gln Leu His Tyr Val Gly His Ser Gln Gly Thr Thr

Ovaries from last instar larvae were transplanted into male larvae; of the same age, so that the donor ovary would have the chance to go through the same developmental events (pupation, adult initiation) as in normal development. The only difference would be the sex of the host and absence of female specific physiological factor (s) if any.

In *Galleria,* the female and male last instar larvae do not exhibit any distinguishable morphological features. The external sex specific features become observable only after pupation. Thus, the host or the donor has 50% chance of being either female or male when transplants are done. Only by examining the shape and the internal anatomy of the gonads from the larvae it is possible to identify the sex of the larvae. Since, female pupae are usually larger than the males, we tested whether the weight of the last instar larvae could be used as a parameter for differentiating male and female last instar larvae.

Day five (D5) last instar larvae were weighed. Two groups were selected, one whose individual weight was 260mg or above and those that were 240mg or below. After pupation the sex of the animals were determined by examining the external features. The data presented in table 5, shows that 92 % of the larvae weighing < 250 mg were males while 97% of the larvae weighing > 250 mg were females. Thus the weight on day five of the last instar can be used as a parameter for enriching for the male or female insects at this stage.

The male hosts with transplanted ovaries that survived the surgery (80%) were allowed to pupate and develop to adults. Their development was not affected by the presence of ovary inside the abdomen. There was a delay in pupation from the normal 4 days to 8 days. However since, sham operated larvae without transplant were similarly delayed in pupation, the delay was attributed to the surgery. Once pupated, the animals developed at the normal rate and the adult emerged by of day eight to nine of pupation as for normal control males. The adults were dissected to see whether the transplanted ovaries have developed. The testis and seminal vesicles of the male hosts were well developed

Table 5: Correlation between weight and sex of Last instar larvae.

D5 L.I larval Weight	#	Sex of Pupae		Percentage
		Female	Male	
>250 mg	50	48	2	96% Females
<250 mg	50	4	46	92% Males

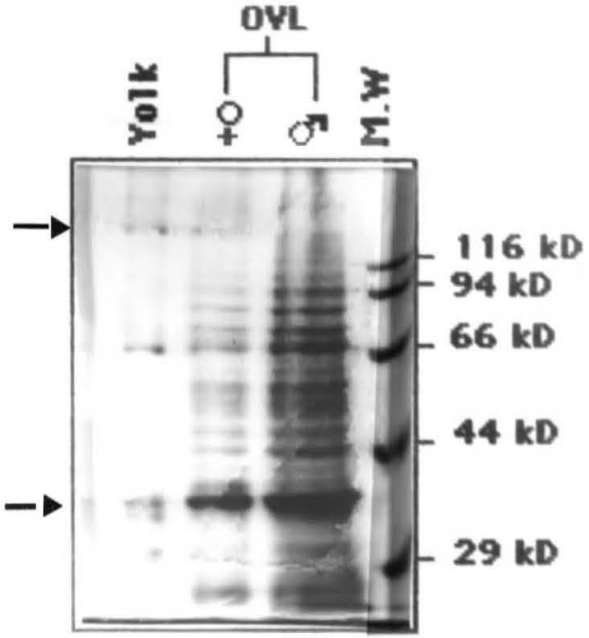
Figure 20: Ovaries Transplanted into males

a) A male host dissected out to show the well developed ovarioles with yolk filled egg follicles (0.2mm). The seminal vesicles marked by the arrow in the middle and lower photogragphs appear normal. The bottom photograph shows the seminal vesicle of a host fused to an ovariole. The fat body cells appear to be similar to those in normal males.

b) SDS/PAGE Analysis of Male Incubated Ovarian Protein Extract.

Proteins extracted from yolk platelets (lane-1), normal ovariole (lane-2) and ovarioles developed in male host (lane-3) were run on 8.75% SDS-PAGE. Lane-4 shows the protein molecular weight markers. The arrows indicate the YP1 and YP4 yolk polypeptides.

b



MALE TRANSPLANTED OVARIOLE PROTEINS
SDS/PAGE

(Fig.20a). 36% (9/25) of the survived hosts contained transplants which were found to be attached to the seminal vesicles in some insects. 60% of the transplants developed quite normally with respect to number of ovarioles per ovary, the size and the number of follicles per ovariole (fig.20a). The oocytes contained opaque yolk deposition although the yellow color was not as intense as the normal eggs. The follicle cells and nurse cells appeared normal. Another interesting feature was that the fat body cells of the male hosts are abundant and formed grape like bunches just as in the normal males. In female adult moth, fat body cells are fewer and smaller in size.

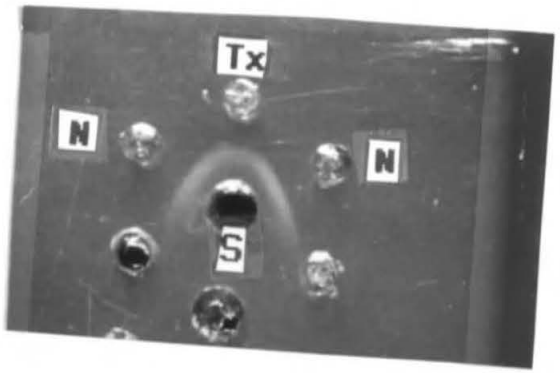
The ability of ovarioles developed in males to express the follicle specific genes and accumulate their products was determined. Protein profiles of normal ovariole and transplanted ovarioles show that the YP4 is expressed at comparable levels (fig.20b). However, the fat body specific YP1 yolk polypeptide in the transplanted ovarioles was found to be less abundant than in normal ovarioles while the YP2 and YP3 polypeptide bands were seen at normal levels (fig.20b, lane 3). The identity of the 37kDa, YP4 band from the transplanted ovaries was further confirmed by Ouchterlony double immunodiffussion with the YP4 antisera. Precipitin lines formed by both the normal and transplanted ovary YP4 (37 kDa) were continuous and did not form any spurs (fig.21a). thus confirming that the 37kDa protein found in the transplanted ovaries is YP4.

To detect the expression of F20 messages in the transplanted ovaries, northern blot analysis was done. Poly (A)+ RNA (0.5 ug/lane) from normal ovarioles and male transplanted ovarioles were size fractionated in formaldehyde denaturing gel and hybridized to nick translated, Cla I / Sty I fragment from F20 cDNA. As figure.20b (lanes N & Tx) shows, F20 transcripts are expressed at normal levels in the male transplanted ovarioles (fig.21b). The results suggest that oogenic development can be autonomous, and that ovaries transplanted into males do not require any female specific factor (s).

Figure 21 a: YP4 yolk polypeptide expression in male incubated ovarioles is confirmed by Ouchterlony double immunodiffusion analysis

Ouchterlony plate in which the antigen YP4, from normal ovarioles (N) and male incubated ovarioles (Tx) were compared by reactions with antibodies (S) produced against the isolated protein, loaded in the center well. Undiluted antisera (20ul) was loaded in the center well. YP4 from an equivalent of 8 to 10 egg follicles are loaded per antigen wells. The single precipitin line formed by the male and normal ovariole YP4 was fused without any spur formation.

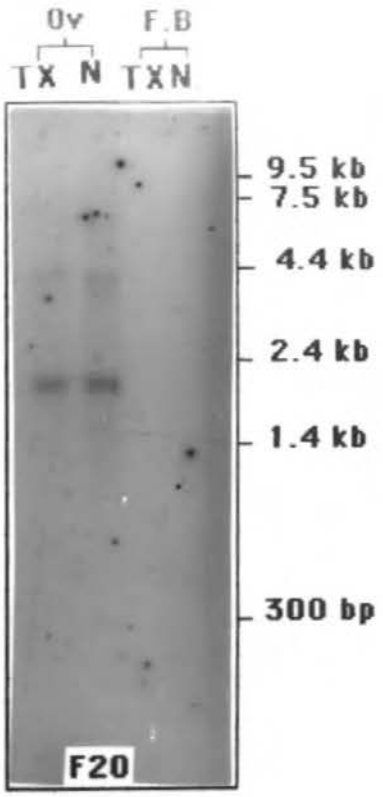
Ouchterlony Double immuno-diffusion



with anti-YP4 antisera

Figure 21 b: Northern analysis showing that F20 message is expressed at normal levels in male transplanted ovarioles.

Autoradiogram of a northern blot analyses of 0.5ug poly A+ RNA/ lane from normal (N) and male transplanted (Tx) ovarioles (OvL) and fat body (F.B) hybridized with nick-translated α^{32}P-dCTP labeled Cla I/ StyI fragment of F20 cDNA. Stringent conditions (65°C) were used for hybridization and washing. Size of the RNA ladder markers are shown.


Ov
F.B
TX N TXN
9.5 kb
7.5 kb
4.4 kb
2.4 kb
1.4 kb
300 bp
F20

In vitro culture of ovarioles

In order to see whether the ovaries can progress into vitellogenesis in the absence of any extra-ovarian factors, they were cultured *in vitro*. In *B. mori* and *G. mellonella*, ecdysone was shown to promote differentiation of follicles in last instar ovaries when cultured *in vitro* (Shibuyi and Yagi, 1975). Since this suggests that ecdysone might be necessary to promote differentiation and make the tissues capable of autonomous development and initiation of vitellogenesis, one set of ovaries were cultured in the presence of ecdysone (3 μg/ml).

Day zero pupal or day three, day four pharate adult ovaries were dissected and cultured with or without ecdysone in the medium under 100% oxygen atmosphere. Ovaries from day zero pupae were used to verify whether the pupal molt is the signal that initiates the oogenic developmental program. Ovaries from day three pharate adult, which have experienced the initiation of adult development, were used to see whether adult initiation is the developmental event (or signal) that induces the oogenic program. For each stage, ovaries from at least three different insects were used.

Four to five days into the cultivation, ovaries in the medium with ecdysone showed some changes. Initially they became transparent and by the sixth to ten days they enlarged in size. The tubular nature of the ovariole with groups of large cells became apparent even though they did not exfoliate from the gonad. Particularly interesting changes occurred in the ovaries that were cultured for three days in ~ 45 to 60 ug /ml ecdysone followed by medium without ecdysone. They enlarged very much and by a week produced four bulb like structures (protruding through the gonad) which appeared to be an imitation of exfoliation of the ovarioles. They displayed and maintained very active contractions for a week without further progression into vitellogenesis. No contractions were observed in ovarian cysts cultured in the absence of ecdysone. Day 3 or 4 ovarioles which have passed the adult initiation event and have exfoliated did not enter vitellogenesis -even after four days in culture, in the presence or absence of ecdysone. The results is tabulated in table 6.

Table 6: In Vitro culture of ovaries from pupae and pharate adults

Culture	Age of pharate adult				Days in Culture	Stage of Terminal Follicles	mRNA Expression		
	D0			D3					
	#	Stage		#			F11	F20	N23
-Ecdysone.	10	S1	S1-3	6	Seven	No Progress	-	-	+
+ Ecdysone (3ug/ml)	10	S1	S1-3	6	Ten	No Progress	-	-	+
+ Ecdysone (45ug/ml)	2	S1			Fourteen	S2 Mimic Exfoliation			

No opaque yolk deposition was observed in the terminal oocytes even after one week in culture. While N23 was expressed, YP4 or F20 transcripts were not found on RNA dot blots.

These observations suggest that the ovary does not progress into vitellogenesis autonomously. While incubation with ecdysone confirms the earlier finding that ecdysone is necessary for normal growth and differentiation of ovaries it is not sufficient to induce the transition from previtellogenic stage to vitellogenic stage. This experiment suggests that ovarian development and vitellogenesis require a factor(s) which probably are produced by ovarian interaction with some other, non sex-specific adult tissue (s).

DISCUSSION

The isolation and characterization of three ovary specific cDNA clones, F11(Ov 11), F20 (Ov5) and N23 (Ov23) were described in the preceding pages and the data are summarized (see Appendix, pg 160). The transcript sizes and the size of fragments to which F11 and F20 hybridize in genomic Southern analysis show clearly that N23, F11 and F20 cDNAs represent three different ovary-specific genes. In *in situ* hybridization, F11 and F20 cDNA probes hybridized only to the columnar follicle cell layer surrounding the oocytes while N23 hybridized only to the nurse cells in the vitellogenic follicles. The squamous follicle cells surrounding nurse cells did not hybridize to any of these probes. In addition, the N23 transcripts were present begining at previtellogenic stage through all oogenic stages and in early embryos. These observations suggest that N23 may represent a maternal message. Identification of N23 gene and its function requires complete nucleotide sequence information, the distribution of its protein and the effect of its inactivation. Nucleotide and deduced amino acid sequences facilitated the identification of F11 and F20 cDNAs. F11 cDNA encodes the *Galleria mellonella* YP4 yolk polypeptide and F20 probably codes for the YP2-yolk polypeptide. Finally, the structure and regulation of the follicle specific yolk polypeptide-YP4 gene was analyzed, and data were obtained for the genes coding for F20 and N23. The significance of these observations in reference to evolution of follicle specific yolk proteins and their regulation will be examined in this section.

F20 cDNA potentially codes for the *Galleria* YP2 yolk polypeptide

The 1614 bp F20 cDNA has a single open reading frame (fig. 16), the direction of which was also confirmed by antisense RNA hybridization (Fig. 17). Starting from the very 5' end of the cDNA it can potentially code for a 504 amino acid polypeptide. *In situ* hybridization of the F20 cDNA (fig.8b) together with the developmental northern analysis

shows clearly that only the vitellogenic follicle cells are the site of expression of these mRNAs, as can be expected of follicle specific YP2 gene expression in *Galleria.* The developmental profile of F20 mRNA (2.0 kb) accumulation is also consistent with the expression of YPs during vitellogenic stages S5 - S9. Considering the transcript length and the potential sites for post translational modifications, the F20 cDNA could code for the 74 kDa YP2 yolk polypeptide.

The deduced amino acid sequence of *Galleria* F20 showing 42% amino acid positional identity with *B. mori* follicle specific yolk protein ESP, and 33 to 29% identity with mammalian lipases (fig. 19 ii,a &b) further support the hypothesis that F20 codes for *Galleria* YP2 yolk polypeptide. The F20 cDNA is incomplete as indicated by the absence of a translation start at the 5' end. Based on the size of the transcript, the similarity (42%) of F20 deduced amino acid sequence when aligned with the follicle cell produced *B. mori* egg specific protein (ESP; 559 aa) and the similar sizes of these yolk proteins (~70 kDa), F20 is probably missing at least another 45 amino acids at the N-terminal end.

Evolution of the follicle specific yolk proteins

Interestingly, *Galleria* F20 deduced amino acid sequence and *B. mori* ESP (Sato and Yamashita, 1991) contain a region that shows 68% identity (11 /16) to the conserved lipid binding domain of human and rat triacyl glycerol lipases. The mammalian lipases show homologies to each other ranging from 28 % to 70 % and have a highly conserved lipid binding domain (Komaromy and Schotz, 1987). *Drosophila* yolk proteins also show 29 - 35% homology to several lipases (Tepstra and Greet, 1988), with strongest similarity shown between YP1 and human lipoprotein lipase (Baker, 1988). In addition *Drosophila* YPs also have a 16 amino acid long conserved lipid binding domain which shows 50% identity to human lipoprotein lipase. The high conservation of the presumed interfacial lipid binding site, suggests that F20, *B. mori* ESP and *Drosophila* YPs have some

common biological properties. Although, the serine residue conserved in the lipases is present in *B. mori* ESP, there is no report of lipolytic activity. This serine residue in the conserved lipid binding region of the lipases, is replaced by asparagin in *Drosophila*, and by aspartic acid in F20 (figure 19 ii b) indicating that the yolk proteins are not catalytically active as the lipases. Since the moth follicle specific proteins, *Galleria* F20 and *B. mori* ESP do not show significant similarity to *Drosophila* YPs, but show the same degree of overall similarity (30 to 35%) to different members of the lipase gene family (which show homology from 28% to 70% to each other) it is likely that these moth and fly yolk proteins evolved independently from different members of an ancestral lipase gene family.

Ancestral lipase genes are believed to have many introns (as many as 14) and *Drosophila* yps are proposed to have evolved by loss of introns/exons and exon shuffling (Kirchgessner, 1989). In *Drosophila* and other higher dipterans the yolk proteins are ~50 kDa in size and the genes contain one small (40 - 60 bp) intron. The apparently larger size of *Galleria* F20 may indicate that the gene may have acquired exon (s). In addition, the similarity to lipases in the F20 deduced sequence begins only after ~120 amino acids. In fact *Drosophila* has a 44 amino acid sequence interrupting the lipase homology alignment and is believed to have acquired an exon and lost the introns (Kirchgessner, 1989). Since the results from this study indicate that F20 has introns and probably belong to a small gene family, the structural analysis of F20 gene should help our understanding of the evolution of this unique group of follicle specific genes in insects.

The apparent molecular weight of the YP4 from egg/yolk extracts based on migration in poly acrylamide gels is 37 kDa (fig.11a, lane7; 11b, lane 3). The calculated molecular weight from the deduced amino acid sequence of F11 is 31.9 kDa. The difference could be accounted for by the post translational modifications such as glycosylation and phosphorylation. There are two adjacent potential N-glycosylation sites N-N-S at amino acid 122-124 and N-S-T at amino acid 123-125 (fig.10, shadowed) one of which could be glycosylated. Infact, digestion of YP4, with endoglycosidase-H which

cleaves the N-linked oligosacharide side chain produced slightly faster migrating polypeptide (data not shown) of ~34 -35 kDa. A serine rich region found between amino acid 97 to 113 in the deduced amino acid sequence, though not homologous to the serine rich domain reported for chicken vitellogenins (Nardelli et al, 1987) and the egg specific protein (ESP) of *B mori* (Inagaki and Yamashita, 1989) is a potential target for phosphorylation. In addition, the region between amino acids 167 and 201 composed of 40% serine and threonine similar to the one found in ESP of *B. mori,* could also be phosphorylated (and/or O-glycosylated). The F20 cDNA also has four potential N-glycosylation sites at aa 63- 65, 71-73, 465-467, 501-503 and a serine rich region between amino acids 22 to 45 (45%), that could be a potential phosphorylation target site, typical of vertebrate and invertebrate yolk proteins (Takanishi et al, 1987). However, F20 is not as serine/threonine rich as the ESP or YP4. In *Xenopus* vitellogenins and *B. mori* ESP (Gottlieb and Wallace, 1981; Sato and Yamashita, 1991) phosphorylation is believed to be neccessary for membrane transport during secretion. Similarly, phosphorylation could be coupled with secretion process of the follicle specific yolk proteins, YP4 and F20 (putative YP2) in *Galleria.*

Comparison of YP4 to the protein sequences in the data bank SWISSPROT* using FASTA program showed similarity to Spherulin-2A, an encystment stage specific glycosylated, protein from the slime mold *Physarum polycephallum.* with 35.2% identity in a region of 213 amino acids (Bernier et al, 1987). The similarity including the conserved amino acid substitutions is 74% (figure.19, i). The positions of the two adjacent N-glycosylation sites and four prolines (figure 19 i, double lined) are conserved and may indicate some similarities in the secondary structure of these two proteins. Apart from the fact that both YP4 and spherulin are produced at a specific stage in tissues associated with reproduction, the functional significance of their similarity is not known, since these two organisms diverged a billion years ago.

The YP4 deduced sequence did not show significant sequence similarity to ESP or other yolk proteins. But it is interesting that YP4 with its high Glx (Glu + Gln) content of 13.9 resembled *B. mori* ESP, Vtg and boll weevil *A. grandis* Vtg (see appendix for Table) and other yolk proteins. F20 / the putative YP2 also has a Glx content of 12.6 higher than average eukaryotic protein. High Glx content is a feature of most vitellogenins (Byrne et al, 1989) and appear to be also true of follicle specific yolk proteins.

Yolk protein genes usually belong to a small gene family probably as an adaptive strategy for mass expression of a single protein in a short period of time without selective DNA amplification (Byrne et al, 1989). In *Galleria* , utilization of yolk protein genes two of which (yp1 and yp3) are expressed in the fat body and two (yp2 and yp4) in the follicle cells to contribute to the yolk may represent another strategy adapted by some lepidopterans. In *B. mori, H. cecropia* and *M. sexta* also two fat body specific yolk proteins (Vg-H and Vg-L) similar to *Galleria* YP1 and YP3 are present. From this study, the follicle cell specific YP2 of *Galleria* appear to be related to *B. mori* ESP. The follicle specific paravitellin of *H. cecropia* is also ~70 kDa in size and probably related to YP2, ESP and the lipases. Since, vitellogenesis occurs in lepidopterans at the late pharate adult stage when the fat body cells are active in the processing of the contents of specialized storage vesicles containing larval hemolymph proteins (see Locke, 1980), their yolk protein synthetic and secretion capacity may be limited. This could be a reason for the additional yolk protein synthesis in the follicle cells in lepidopterans. However, it is interesting that a follicle specific yolk protein similar to *Galleria* YP4 is not reported in *B. mori, H. cecropia or M. sexta.* Based on sequence data from this study *Galleria* YP4 appears to be a novel yolk protein not related to other yolk proteins studied so far, except for similarity in the amino acid composition. The higher serine/ threonine content probably indicates that YP4 also acts as phosphate reservoir and nutritional source.

The question arises also as to the site of expression of the ancestral YP genes. In some dipterans such as *Stomoxys* (Chen et al,1987) all yolk proteins (~ 50 kDa) are

synthesized only in the follicle cells. In *Drosophila*, on the other hand, all three yp genes (yp1, yp2, yp3) are expressed in the fat body and follicle cells. In *Galleria* , F20, the putative YP2 which is the *B.mori* ESP (72 kDa) and mammalian lipase homolog, is expressed only in the follicle cells along with YP4. It is not known whether the ability to express these genes in the fat body cells and follicle cells is a derived feature or evolutionarily ancestral state.

It is interesting that two modern vertebrate serum proteins, apolipoprotein B-100 of human LDL (low density lipoprotein) a transporter of cholesterol (and triacylglycerol) and the enzyme, lipoprotein lipase which has a functional role in the conversion of VLDL to LDL, contain domains that are similar to parts of vitellogenins from *C. elegans* and insect *Drosophila* (Baker, 1988). Commandeering the use of lipase genes to serve as yolk proteins may be related to their lipid binding property, to store ecdysone, a steroid hormone in insect eggs. In *Galleria* , ovarian follicles were shown to produce ecdysteroids (Bollenbacher et al, 1978). In *Locusta migratoria* the conjugated ecdysteroids, present in the eggs appear to be synthesized in the follicle cells (Lageaux et al, 1987) and are stored in the egg. Lipid binding domains of the follicle specific yolk polypeptides, F20 in *Galleria* as well as ESP in *B. mori* probably help transport ecdysteroids produced in the follicle cells store them in the eggs to be released upon proteolysis during embryogenesis. In *Drosophila*, YPs were shown to be tightly bound to apolar conjugates of ecdysteroids and release them upon proteolytic cleavage (Bownes et al, 1988). In *Locusta* also, the yolk proteins were shown to be responsible for regional localization of maternal ecdysteroids in the egg (Lageaux et al, 1984). F20 with its lipid binding domain most likely functions in a similar manner. Thus, the idea and evidence that yolk proteins might have additional function(s) at least for a limited time is slowly emerging. Specific functional domains; a Cys-His motif for metal binding in *B. mori* ESP (Inagaki and Yamashita, 1989) and lipid or ecdysteroid conjugate binding in *D. melanogaster* (Bownes et al, 1989) and locust (Lageaux et al, 1977) YPs have been reported. Further more, the *B. mori* ESP was

shown to be cleaved at specific sites by a specific protease during embryogenesis (Sato and Yamashita,1989) to release specific peptides that may have specific functions. As shown in this study, when ovary was transplanted into males, eggs developed and follicle specific yolk protein genes, YP2 and YP4 were expressed at normal levels. Infact in *B. mori*, embryo developed normally and larvae hatched from eggs developed in males (Yamashita and Irie, 1984). Since the fat body specific yolk protein was at trace levels in these eggs, this shows that follicle specific yolk proteins are sufficient to support normal oogenesis and embryogenesis.

Regulation of moth oogenesis

In all insects, a discrete period of previtellogenic development must be completed before vitellogenesis. How previtellogenesis to vitellogenesis transition is achieved in insects is an interesting question. In unfed adult *Rhodnius prolixus,* a hemipteran, the largest follicle in each ovariole has completed its pre-vitellogenic development and remains in a state of arrest until blood meal. Vitellogenesis is activated by a blood meal and subsequent secretion of JH (Abu-Hakima and Davey, 1977). Similar situation is found also in the anautogenous mosquitoes where blood meal and subsequent production of ecdysone is needed to induce egg development. In *Galleria* and other moths such as *H. cecropia* (Telfer and Smith, 1978) by contrast, the transition from previtellogenic to vitellogenic stages occurs without any external cue.

The absence of developmental arrest of ovarian follicles in these moths may be related to their feeding habits. The pharate adult moths do not feed, and yolk proteins are synthesized from the reserve of nutrients (such as the LHPs) stored in the fat body during the larval life. In the absence of a need for an external nutritional cue unlike in *Rhodnius* and anautogenous mosquitoes, lepidopterans may have developed other strategies to control vitellogenesis. Is vitellogenesis in the moths pre-programmed into ovarian developmental program? If pre-programmed at which developmental stage does the

program become operative? The results reported here on *in vitro* culture of ovaries and transplantation of ovaries into males throw some light on these questions.

The data presented in this report (figure 20 and 21 a, b) show that the male incubated ovaries underwent vitellogenesis confirming the observation of Telfer (1954), Irie and Yamashita (1983) and Lamy (1984). As this study shows, follicle specific transcripts and yolk proteins are expressed at normal levels in male developed ovaries. It is interesting that the eggs developed in males had only trace amounts of the 159 kDa YP1, while the levels of the ovary specific yolk polypeptides YP2 and YP4 and the fat body specific 44 kDa, YP3 yolk polypeptides are nearly normal (Fig.-20 b, lane 3). Ouchterlony double immuno diffusion analysis (Fig.21a) also confirms that the male developed oocytes contain the follicle specific yolk protein YP4. The F20 (Fig.21 b, lanes 1 and 2) and F11 (data not shown) transcripts in the transplanted ovaries were as abundant as in normal ovaries. Thus, even with reduced levels of fat body specific yp1 expression the other three yolk protein genes are expressed. In eggs produced in male *B. mori* (Yamashita and Irie, 1984) and *H. cecropia* (Telfer, 1954) also the fat body produced yolk proteins were at trace level. Also in *B. mori*, the follicle specific ESP was shown by Ouchterlony double immuno diffusion analysis, to be at same levels as in normal eggs.

The fact that YP1 and YP3 are independently variable suggests that YP1 and YP3 may be produced from two independently regulated genes. If this were to be the case the wax moth fat body specific yolk proteins are different from those of locusts (Locke et al, 1987) mosquitoes (Hagedorn, 1990) and boll weevil (Trewitt et al, 1992) in which the so called large and small subunits of the Vgs are coded for by the same gene. In *Galleria*, limited N-terminal amino acid sequence of YP1 protein has been obtained (Appendix) to facilitate the isolation of a yp1 genomic clone. Since, in *Bombyx* two separate mRNAs (Izumi and Tomino, 1983) were shown to code for the two fat body specific yolk proteins, it is reasonable to suggest that this feature may be common to many lepidopterans.

For the transplant study Day 6 or 7 last instar larval ovary was transplanted into same stage male host. Thus the ovary was exposed to all the developmental cues (eg; larval/pupal molt, adult initiation) as in normal development. Results reported here show that the YP4 and F20 are expressed at normal levels when ovaries transplanted into same age male host, suggesting that all the developmental cues for initiation of oogenesis are present in males. Transplantation of larval ovaries into day 1 or day 2 pharate adults (before exfoliation of ovarioles) or day 3 to 4 (after exfoliation) pharate adults would help to identify the specific developmental signals (or factors) necessary to initiate oogenesis. However, since the survival rate of pharate adults is very low after surgery, improvements in the surgical procedures would be necessary before these questions can be answered.

The observations reported here on *in vitro* development of ovaries confirm the previous reports by Shibuya and Yagi (1972), that ecdysone is necessary for differentiation of the ovarioles. They used late last instar larval ovarian cysts in the *in vitro* culture experiments and reported the differentiation of ovarian follicles to S3, in the presence of ecdysone (3 to 5 ug / ml) and suggested that ovaries from insects of later stages, might be able to proceed autonomously into vitellogenesis when cultured in vitro. However, in the *in vitro* experiments with ovarian cyst from day zero pupae (ie; ovaries that have experienced the developmental signals associated with pupation) and from day 2 or 3 pharate pupae (ie; ovaries that have experienced the developmental signals of adult initiation) in the presence or absence of ecdysone vitellogenesis did not begin, indicating that extra ovarian factors are involved. Alternatively the stage of ovarian follicle that become committed may be later than the stages used (S3 and early S4). Since the YP4 and F20 messages are expressed by S5 the commitment stage probably occurs within a short period at S4. Involvement of some extra ovarian factor(s) is suspected also in *B. mori* in implementing the vitellogenic (Tsuchida, 1987) and choriogenic programs (Swevers et al,1992). The normal development of ovary and gene expression in the male suggests that this factor is available in the male and is not sex specific.

Head ligation as well as prothoracic ligation of day zero *Galleria* pupae was found to block initiation of adult development as well as oogenesis (unpublished observations). There may be a requirement for a neurohormone from the brain after pupation (D0), probably released during the initiation of adult development (~36 hrs after pupation). The *in vitro* ovarian culture experiments suggest that ecdysone alone is not sufficient. It is possible that the ovary interacts with the brain of the male host and induce the production of a neurohormone. In *Aedes aegypti* an egg development neurohormone (EDNH) has been found to induce egg development (see, Hagedorn, 1985). In *Rhodnius*, an antigonadotropin, a neuropeptide from peripheral neurosecretory organs in the abdomen was found to antagonize the action of JH on the follicle cells (Ilenchuk and Davey, 1987). Such gonadotropins and antigonadotropins may be widespread among insects. Recently, an antigonadotropin is shown to function in *Locusta migratoria.* (Davey et al, 1993). Coculture of ovary *in vitro* with other tissues such as the fat body, brain, other ganglia or gut should give us some clue as to the source of the extra-ovarian factor in *Galleria.*

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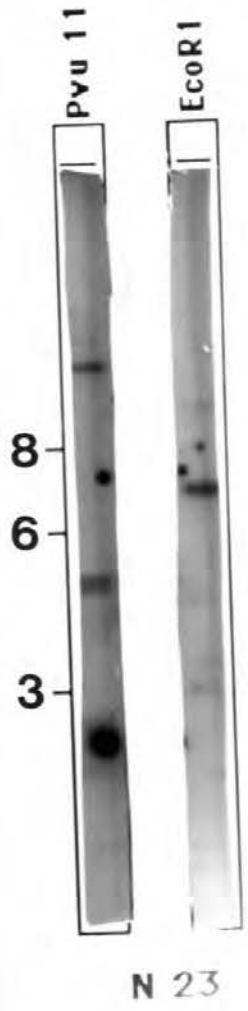
APPENDIX

Summary: Galleria ovariole-specific cDNAs

	N23	F20	OV11
Insert size	2.0 kb	1.6 kb	0.95 kb
Transcript Size	2.3 kb	2.0 kb	1.1 kb
Developmental Stage Specificity	All oogenic stages + embryo	Vitellogenic stages only	Vitellogenic stages only
Cell Type Specificity	Nurse cells	Follicle cells	Follicle cells
Gene Copy #	Single copy	Single copy or Small gene family	Single copy
Gene Structure	Introns present	Introns present	No Introns in coding region
Identity	Maternal message	Putative YP2	YP4 !
Similarity	---	B. mori ESP Triacylglycerol lipases of mammals	Spherulin 2a slime mold encystment protein

Genomic Southern analysis with N23 cDNA indicate that it represent a single copy gene with introns

Autoradiograms of Southern blots with a) 10 ug/lane genomic DNA, digested with PvuII or EcoRI were size fractionated on 0.6% agarose gel in TAE buffer.



YOLK PROTEIN 1 [YP1] N-TERMINAL AMINO ACID SEQUENCE

HIS LEU SER LYS SER PHE HIS ILE ASP LYS MET ILE VAL ASN GLN

Antisense oligomer: 5'- GAT AAA ATG ATC GTX AAT CAA -3'
C G T G
A

Amino acid composition of *Galleria* Yolk polypeptide-YP4, F20 and yolk proteins from *B. mori* and *A. grandis.*

AA	YP4	F20	ESP*	B. m / Vg	A. g / Vg**
Asx	8.0	7.3	12.9	11.1	10.2
Thr	7.9	4.2	5.1	5.7	6.0
Ser	9.8	7.9	8.2	6.5	7.7
Glx	13.9	12.6	11.1	13.9	16.3
Pro	3.8	5.8	2.5	5.3	4.1
Gly	6.7	5.8	9.3	5.9	4.3
Ala	6.3	5.6	7.9	7.8	4.8
Val	7.7	6.5	5.5	6.9	6.8
Met	0.7	5.6	1.3	1.7	2.7
Ile	5.6	4.2	4.0	5.9	5.7
Leu	7.3	7.1	7.8	7.8	7.4
Tyr	4.2	7.3	1.7	2.0	4.1
Phe	3.5	2.6	5.9	4.9	3.5
His	2.1	4.2	6.2	7.0	2.4
Lys	4.2	7.5	5.7	3.2	7.3
Arg	4.2	4.6	3.4	4.4	5.0
Cys	1.4	0.6	---	---	1.1
Trp	2.4	0.8	---	---	0.9

* Irie and Yamashita (1983)

** Trewitt et al (1992)