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SEQUENCE ANALYSIS OF A FOLLICLE CELL-SPECIFIC GENE FROM THE MOSQUITO, *AEDES AEGYPTI*

by

Yonggu Lin

A Thesis Submitted to the Faculty of the
DEPARTMENT OF ENTOMOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
MASTER OF SCIENCE
In the Graduate College
THE UNIVERSITY OF ARIZONA

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Henry H. Hagedorn — April 25, 1991

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ABSTRACT

The follicle cell-specific (FCS) gene, a 3,023 bp gene specific to follicle cells of the mosquito, *Aedes aegypti*, was characterized at the nucleotide level. Genomic Southern blots demonstrated that there was only one copy of this gene in the *A. aegypti* genome. An ovary-specific cDNA library was constructed from female mosquitoes 24 hours post blood meal. Then a cDNA clone containing the complete coding region was identified, and its nucleotide sequence was determined. The deduced protein contained unusually high levels of alanine and proline. Search of a protein data base revealed that the FCS gene was similar to *Drosophila* vitelline membrane protein genes, with 47.5% similarity in nucleotide sequence and 46.7% similarity in amino acid sequence. The conserved hydrophobic regions from several vitelline membrane proteins were compared. Another cDNA clone, 1D, was isolated from the cDNA library screened with the FCS gene. These two mosquito genes shared a 60% similarity at the nucleic acid level and a 79.8% similarity at the amino acid level.

INTRODUCTION

Egg Development in the Mosquito, Aedes aegypti

Aedes aegypti is an anautogenous mosquito, whose egg development commences upon emergence of the adult as the oocyte grows and differentiates from the nurse cells. This results in resting stage follicles, which will complete ovarian development after a blood meal (Gillett, 1956).

A. aegypti has a pair of spindle-shaped ovaries. Each ovary comprises approximately 60 polytrophic ovarioles, and at the time of adult eclosion each ovariole consists of a primary follicle and germarium. Each follicle consists of an oocyte and seven nurse cells, enveloped by a layer of follicular epithelial cells (Laurence, 1977). The primary follicles begin to mature at adult emergence. A secondary follicle becomes visible in each germarium three days after adult emergence and prior to the first blood meal (Beckemeyer and Lea, 1980).

The ovarian development of *A. aegypti* can be divided into the four stages microscopically. The first of these is the previtellogenic stage in which the oolemma bears a dense coat of microvilli (Roth and Porter, 1964) and the follicle cells seal the oocyte from the hemolymph before blood meal. The second stage is the micropinocytotic stage when numerous microvilli appear and receptor mediated endocytosis of vitellogenin is substantial. In the third stage, vitelline membrane is deposited between the follicle cell layer and the

oocyte, and pinocytosis ceases. Finally, during the chorionic stage the egg shell is laid down (Anderson and Spielman, 1973).

Clements and Boocock (1986) examined the mosquito ovary developmental gates by vital staining with neutral red. Developmental gates are points of potential arrest. At these gates follicular and oogenic development will be halted unless the appropriate stimulus is given. They described the mosquito ovarian development process as germarial gate, stage I gate, previtellogenic gate, stage III gate, and maturation gate. The germarial gate occurs immediately after adult emergence in *A. aegypti.* The follicle has partly separated from the germarium, and the internal stimulus for this gate is ecdysterone. At the stage I gate, the follicle has separated from the germarium, the oocyte is entirely surrounded by follicular epithelium, and the oocyte is distinct from the nurse cells. The stage I gate is three days after adult emergence in *A. aegypti*, and the JH is the stimulus. The previtellogenic gate is at age 3-5 days in *A. aegypti.* The stimuli for this gate are egg development neurosecretory hormone (EDNH) and ecdysterone. The stage III gate happens after the mosquito completes a blood meal. Yolk deposition in the ooplasm begins, the oocyte grows dramatically, and the eggshell starts to appear. The stimulus for this gate is ecdysterone again. At the maturation gate, the oocyte grows to full length. The follicular epithelium degenerates, and chorionic structures, such as surface sculpturing are fully formed. This is about 72 hours post blood meal in *A. aegypti.*

Yonge and Hagedorn (1977) have investigated the timing of entry of vitellogenin into the oocyte after a blood meal using trypan blue as an

indicator of vitellogenin uptake. They have shown that vitellogenin starts to be taken up between 2 to 5 hours, peaks at 24 hours, and terminates at 39 to 42 hours after a blood meal. The follicles keep growing after the termination of vitellogenin uptake until the eggs are laid.

The major yolk protein, vitellin, is derived from vitellogenin synthesized by extraovarian tissues. Ouchterlony immunodiffusion assay reveals that vitellogenin is a female specific protein (Hagedorn, 1974). By immunoprecipitating with antiserum against yolk protein, Hagedorn and Judson (1972) have demonstrated that vitellogenin is synthesized in the fat body. The site of vitellogenin production has been further confirmed by *in vitro* organ culture of fat body preparations (Hagedorn *et al.*, 1973). Vitellogenin consists of two subunits of 200 kDa and 65 kDa (Raikhel and Bose 1988), and both subunits are derived from a single precursor (Bose and Raikhel, 1988). The vitellogenins are transported via hemolymph, then enter the follicle through the intercellular spaces of the follicle cells, accumulate in the perioocytic space, and are sequestered into the oocyte by micropinocytosis (Roth and Porter, 1964). Furthermore, by means of an *in vitro* assay, Koller *et al.* (1989) have demonstrated that vitellogenin is accumulated in oocytes by receptor-mediated endocytosis with features of temperature dependence, saturability, selectivity and tissue specificity.

Control of Oogenesis in Mosquitoes

A. aegypti oogenesis requires the stage and tissue specific expression of several kinds of proteins. Differential gene expressions may be regulated at many levels of information flow.

Reproductive maturation in *A. aegypti* is under the command of several hormones, each of which has a distinct time course of induction and regression. Blood meals may induce a gonotrophic cycle in anautogenous mosquitoes. Upon the intake of a blood meal, juvenile hormone (JH) levels decrease (Shapiro *et al.*, 1986), EDNH is released (Lea, 1972; Hanaoka and Hagedorn, 1980; Greenplate *et al.*, 1985), and 20-hydroxyecdysone levels increase (Hagedorn *et al.*, 1975; Greenplate *et al.*, 1985).

20-hydroxyecdysone is one of the key hormones in directing ovarian development in *A. aegypti.* Specifically, ecdysone released from mosquito ovaries stimulates the synthesis of vitellogenin in fat body (Hagedorn and Fallon, 1973). In addition, feeding or injecting ecdysterone in unfed mosquito females induces yolk deposition (Spielman *et al.*, 1971; Fallon and Hagedorn, 1972). 20-hydroxyecdysone stimulates fat body from unfed females to synthesize vitellogenin *in vitro* (Fallon *et al.*, 1974). A sharp rise in the 20-hydroxyecdysone titre shortly after a blood meal in females is positively correlated with the onset of vitellogenin synthesis by fat bodies.

The ovary is the source of ecdysone in adult mosquitoes (Hagedorn *et al.*, 1975). This is supported by the fact that *in vitro* ovary culture responding to a head extract produces ecdysone (Hanaoka and Hagedorn, 1980). Fat

bodies become competent in response to *in vitro* 20-hydroxyecdysone treatment at 56-60 hours after eclosion, and again 96 hours after blood meal (Ma *et al.*, 1988). It has been suggested that the responsiveness to 20-hydroxyecdysone initiation and termination of vitellogenin production in the fat body is pre-programmed (Bohm, *et al.*, 1978; Ma *et al.*, 1987).

20-hydroxyecdysone is normally required but is not the only factor for vitellogenesis process in the mosquito. By injecting a 1,000-10,000 times higher than the physiological level dose of 20-hydroxyecdysone into blood-fed decapitated females, abnormal oogenesis is initiated. Specifically, contents of protein and lipid in yolk are disturbed (Lea, 1982), and an abnormal vitelline membrane is deposited (Raikhel and Lea, 1982).

Neuropeptides from mosquito heads, particularly the EDNH, are responsible for the ecdysteroid secretion from ovaries. The cellular origin of EDNH is the medial neurosecretory cells (Lea, 1967; 1972). EDNH regulates reproduction by activating the ovaries to synthesize ecdysone after a blood meal (Hagedorn *et al.*, 1979). Injection of EDNH into blood fed decapitated females increases ecdysteroid and vitellin levels (Wheelock and Hagedorn, 1985; Matsumoto *et al.*, 1989). Another peptide isolated from mosquito ovaries is claimed to be an oostatic hormone, and is shown to suppress vitellogenesis (Borovsky, 1985). Borovsky (1988) also demonstrated that this hormone inhibits biosynthesis of trypsin-like enzymes, thus regulating digestion of blood meal by trypsin. The amino acids resulting from blood protein digestion are required for oogenesis to occur (Gooding, 1966).

Juvenile hormone secreted from the corpus allatum also plays important roles in the mosquito reproduction. JH titers begin to rise after adult emergence, peak on day 2 then fall slowly to low levels, and climb again two days after blood meal (Shapiro *et al.*, 1986). Physiologically, JH not only stimulates the fat body to become competent to respond to 20-hydroxyecdysone (Flanagan and Hagedorn, 1977), but also promotes the post emergence ovarian development to resting stage (Gwadz and Spielman, 1973). Furthermore, it stimulates the ploidy to increase in fat body after emergence and after blood feeding (Dittmann *et al.*, 1989). JH also induces the mating behavior of female mosquitoes (Lea, 1968; Gwadz *et al.*, 1971).

JH and 20-hydroxyecdysone are both necessary and each plays its specific role in oogenesis of *A. aegypti.* Experiments with decapitated, blood-fed females and with isolated abdomens from blood-fed females suggest that JH also increases the activity of 20-hydroxyecdysone (Borovsky, 1981; Borovsky *et al.*, 1985). Applying 20-hydroxyecdysone and JH analog to isolated abdomens of blood fed females immediately after ligation increases the responsiveness to 20-hydroxyecdysone at 18 hours post blood meal (Martinez and Hagedorn, 1987). *In vitro* studies have indicated that the correlation between JH and 20-hydroxyecdysone is positive (Racioppi *et al.*, 1986).

Hagedorn (1985) has described a basic scheme of egg development in the anautogenous mosquito, *A. aegypti*, and the related tissues and counter reaction of the hormones involved. The process is initiated by a blood meal. Specifically, the taking of a blood meal sends a signal to the brain to release

EDNH. EDNH induces ovaries to produce ecdysone, which is converted to 20-hydroxyecdysone in the hemolymph. 20-hydroxyecdysone stimulates the fat body to synthesize vitellogenins. Finally vitellogenins are taken up by oocytes.

Follicular Epithelium of the Ovary

In *A. aegypti*, the follicle cells replicate after adult emergence, but cease dividing 48 hours later (Laurence and Simpson, 1974). During previtellogenic growth, follicle cells remain tightly attached to each other by desmosomes (Roth and Porter, 1964; Anderson and Spielman, 1971), and are interconnected by cytoplasmic bridges allowing communication between cells (Meola *et al.*, 1977). After a blood meal the follicle cells divide again, and a high mitotic rate is reached at 4 to 5 hours post blood meal (Laurence and Simpson, 1974).

In *Rhodnius prolixus*, vitellogenin reaches the surface of the oocyte via the spaces between the follicular epithelium cells, that surround each oocyte. The reduction of the follicle cell volume results in large spaces between these cells. This status is known as patency (Pratt and Davey, 1972). Patency has been described previously by Roth and Porter (1964) in *A. aegypti*. After a blood meal follicle cells separate from one another. This creates larger intercellular spaces and the number of desmosomes decrease (Roth and Porter, 1964). The follicle is surrounded by a basement membrane, which serves as a mechanical filter to exclude molecules larger than 200-500 Å. Intercellular channels lined with mucopolysaccharide exist between the follicle

cells. Exogenous molecules pass through the follicle epithelium by penetrating the follicle cell channels. Intercellular channels of the follicle cells become occluded by desmosomes 36-48 hours post blood meal (Anderson and Spielman, 1971). In the moth *Hyalophora cecropia*, follicle cells produce a sulfated proteoglycan, which is a matrix to maintain the patency of the peritrophocytic space (Telfer, 1979).

Abu-Hakima and Davey (1977) have demonstrated that patency is controlled by JH in *R. prolixus*. The JH-induced reduction of follicle cell volume is ouabain sensitive. This suggests that Na^+-K^+-ATPase of follicle cell membrane is likely to be the site of hormone action (Abu-Hakima and Davey, 1979). Studies of the characteristics of ATPase from follicle cells show that this enzyme is similar to ATPase in other systems. The ATPase activity from brain is unaffected by JH, indicating that the JH-responsiveness of Na^+-K^+-ATPase from follicle cells is tissue specific (Ilenchuk and Davey, 1982). Applying JH I directly to microsomal preparations of vitellogenic follicle cells increases Na^+-K^+-ATPase activity by activating a specific JH-sensitive Na^+-K^+-ATPase in the membrane (Ilenchuk and Davey, 1983). In addition, JH I binds to the preparations of follicle cell membrane in the nanomolar range in a saturable fashion (Ilenchuk and Davey, 1985).

In polytrophic follicles of *Hyalophora cecropia* and several other insects, nurse cells provide a large amount of RNA to the oocyte. This process is mediated by electrical currents. These currents are generated because the steady state potential of the nurse cells is several millivolts more negative than that of the oocyte. An outward current leaves the follicular epithelium covering

the oocyte, and an inward current enters the follicular epithelium covering the nurse cells (Jaffe and Woodruff, 1979). Therefore, the negatively charged molecules, such as protein and RNA, are able to migrate through intercellular bridges from the nurse cells to the oocyte (Woodruff and Telfer, 1980). The follicular epithelium overlying the trophic cap is a diffusion barrier to extracellular ion movement. This ensures that an exit current originated from the seven nurse cells flows posteriorly to the oocyte through the intercellular spaces of the trophic cap (Woodruff *et al.*, 1986).

A group of follicle cell products which are incorporated into oocyte as yolk protein have been studied in moths and *Drosophila*, for example, egg specific protein from *Bombyx mori* (Irie and Yamashita, 1983), paravitellogenin in *Hyalophora cecropia* (Bast and Telfer, 1976), yolk proteins 1 and 2 from *Drosophila melanogaster* (Brennen *et al.*, 1982), and *Drosophila grimshawi* (Kambysellis *et al.*, 1986). The *Drosophila* "yolk proteins" are not related to the vitellogenin of other insects (Baker, 1988), but apparently play the same physiological role (Bownes *et al.*, 1988). The two yolk proteins 1 and 2 in *Drosophila melanogaster* have molecular weights of approximately 46 and 45 kDa (Warren and Mahowald, 1979).

Eggshell Structure in Insects

Descriptions of the eggshell structure of *Aedes* are not consistent in the literature. The terminologies to be used in this report are defined according to Slifer (1937). Vitelline membrane is a very thin noncellular layer surrounding

the oocyte. Chorion is usually defined as being above the vitelline membrane, and can be distinguished as having an inner layer and an outer layer, which are respectively called endochorion and exochorion.

The major architecture of the eggshell is constructed with the vitelline membrane and chorion. Proteins in the eggshell are produced by the follicle cells (Margaritis, 1985). Interesting insights are obtained into endocrinological regulation of gene expression and evolution of gene families by studies on the synthesis of vitelline membrane proteins and chorion proteins, as well as mechanisms in regulating the switch in follicle cell production of these proteins.

Chorion has been investigated in great detail at the levels of gene structure and organization, gene expression, protein synthesis, protein secondary structure, ultrastructure, and evolution (Regier and Kafatos, 1985). Most of the research emphasis has focused on *Antheraea polyphemus*, *Bombyx mori*, and *Drosophila melanogaster*, with *A. polyphemus* being the dominant model.

In *A. polyphemus*, chorion proteins are very complex. They can be categorized into 5 to 6 groups, labeled A, B, C, D, E, and Hc, according to their electrophoretic behavior in SDS gels (Paul *et al.*, 1972). More than 90% of the dry weight of the chorion consists of proteins. An individual silkmoth can contain as many as 186 different chorion proteins identified by two-dimensional gel electrophoresis (Regier *et al.*, 1980). Chorion proteins have been investigated in several silkmoths and they appear to be related (Kawasaki *et al.*, 1971, 1972; Paul *et al.*, 1972b). The proteins are small, with

an average molecular weight of 11.5 kDa in *A. polyphemus* (Paul *et al.*, 1972a). The proteins are quite nonpolar, having an average of approximately 69% nonpolar amino acid residues (Regier *et al.*, 1978). The purified silkmoth chorion protein is rich in glycine, alanine, cysteine and tyrosine, and poor in methionine and histidine (Regier *et al.*, 1978). These proteins show higher frictional coefficient ratios than a number of globular proteins (Kawasaki *et al.*, 1971, 1972; Paul *et al.*, 1972a), which is consistent with the fibrous ultrastructure of the chorion.

In *A. polyphemus* chorion, the A, B, C, and Hc protein sequences have the same basic organization. Namely, they consist of arms on both sides of a central region. The central region, consisting of β-sheets alternating with β-turn, is conserved in sequence and is more conserved in length in the A and Hc proteins (Homodrakas *et al.*, 1985). By contrast, the arms are characterized by short repeating peptides such as Cys-Gly or some variation on Gly-Tyr-Gly-Gly-Leu (Rodakis *et al.*, 1982). Conservation of the central region also occurs in the B and C proteins. It appears that maintenance of the central region sequence and length is of special importance during the evolution of chorion proteins (Regier *et al.*, 1983). The A proteins have short arms while the carboxyl terminal arms of the B proteins are long; the C proteins have two long arms that may be important in forming the initial framework (Regier *et al.*, 1983). The arms have many cysteines that are involved in cross-linkage.

Chorion proteins are produced in the late period of egg development. *A. polyphemus* choriogenesis can be divided into four morphogenetic modes: lamellar framework construction, lamellar expansion, lamellar densification,

and regionalized aeropyle crown formation (Mazur *et al.*, 1989). Different subsets of chorion proteins in silkmoths are responsible for each of the morphogenetic modes (Mazur *et al.*, 1980, 1982; Regier *et al.*, 1980). Each mode is characterized by the synthesis of a discrete set of proteins during the corresponding period of choriogenesis. The expression of the E1 and E2 genes, coding for E proteins, is typically programmed temporally and spatially (Hatzopoulos and Regier, 1986; Regier *et al.*, 1984). The timing of the chorion protein synthesis has been determined using autoradiography (Paul and Kafatos, 1975). Pulse-chase experiments demonstrate that proteins produced at different times end up in different places in the chorion (Paul *et al.*, 1972).

Disulfide bond formation at ovulation leads to an impenetrable eggshell in *A. polyphemus*. Developing chorions are soluble in 8M urea without any reducing agent. However, solubility changes so dramatically at the time of ovulation that 80% of chorion proteins become insoluble without a reductant (Blau and Kafatos, 1979). Disulfide bonds are formed by covalent cross-linking between cysteine residues. The rules are: late A proteins can only crosslink with late B proteins, and with no others; by contrast, late Bs crosslink with other late Bs as well as late As; E1 and E2 crosslink only with themselves and with each other (Regier and Wong, 1988). In comparison, disulfide bonds are formed by di- and trityrosine cross-linking in *D. melanogaster*.

Regier *et al.* (1983) conclude that chorion gene families constitute a single superfamily. The developmental regulation of silkmoth choriogenesis is a good model to study gene evolution. Evidence indicates that the multiplicity of chorion proteins is due to the multiplicity of structural genes, rather than

post-translational modifications of a small number of primary products (Regier *et al.*, 1978). Hatzopoulos and Regier (1987) hypothesize that evolutionary changes in chorion morphology are the results of regulatory changes in chorion gene expression. By comparing protein synthesis and morphogenesis in *Manduca* and silkmoth, Regier and Vlahos (1988) suggest that changes in pretranslational mechanisms ultimately appear to be responsible for evolutionary changes in morphogenesis.

Chorion gene DNA shows organizational and regulatory conservation. Some of the silkmoth chorion genes exist in pairs in opposite orientation so that the 5' regulatory regions lie between them. This results in close coordination of expression (Jones and Kafatos, 1981; Iatrou and Tsitilou, 1983; Spoerel *et al.*, 1986). E1 and E2 genes are separated by approximately 7.5 kb and are transcribed from the same DNA strand (Hatzopoulos and Regier, 1986). A highly conserved motif within the upstream region, TCACGT, is essential for chorion-specific expression. This hexamer has also been found in *B. mori* (Spoerel *et al.*, 1986) and *Drosophila* (Martinez-Cruzado *et al.*, 1988). Most of the silkmoth chorion genes sequenced thus far have a common cap site sequence ATTAGT, an intron near the end of the signal peptide encoding region, and a Hogness box 21 to 23 nucleotides upstream from the cap site (Regier and Pacholski, 1985; Jones and Kafatos, 1980). Likewise, *D. melanogaster* chorion genes in the third chromosome are organized in this way: a small and a large exon separated by a short intron in the signal peptide region (Wong *et al.*, 1985).

Drosophila has been used for studying developmental regulation because of the availability of a convenient germ-line transformation procedure and a wealth of developmental, genetic, and cytogenetic information. *Drosophila* chorion proteins are less complex than those in silkmoth. In addition, specific chorion genes are amplified during *Drosophila* choriogenesis. Chorion gene organization has been assigned to two clusters: genes on the X chromosome at 7F1-2, and genes on the third chromosome at 66D11-15 (Spradling *et al.*, 1980). Follicle cell and stage-specific amplification of chorion genes apparently occurs to provide enough template for the rapid accumulation of chorion mRNAs during the short period of choriogenesis (Snyder *et al.*, 1986). At stage 8 or 9 of oogenesis, the polyploid follicle cells begin to amplify two clusters of genes that encode the major chorion or eggshell proteins (Spradling and Mahowald, 1980). By the end of choriogenesis, amplification levels have reached a maximum of 20-fold in the sex-linked chorion cluster and 60 to 80-fold in the chorion cluster of the third chromosome (Spradling, 1981). The medfly, *Ceratitis capitata,* has also been found to show chorion gene amplification (Konsolaki *et al.*, 1990).

The Vitelline Membrane Genes of Drosophila

In *Drosophila,* histochemical and ultrastructural evidence demonstrates that follicle epithelium is the site of vitelline membrane protein synthesis during the vitellogenic stage (Mahowald and Kambysellis, 1980). This is further supported by an *in vivo* labeling experiment (Fargnoli and Waring, 1982).

Petri *et al.* (1976) have identified four vitelline membrane proteins according to their amino acid composition, richness in proline and alanine, and stage specific synthesis during the period of vitelline membrane deposition. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two dimensional electrophoresis, Fargnoli and Waring (1982) have identified six major molecular weight classes of vitelline membrane proteins on the basis of selective enrichment in purified eggshell fragment preparations, time and site of synthesis, and selective binding to eggshell antisera containing anti-vitelline membrane activity. Some of the protein species are similar in the two studies by Petri (1976) and Fargnoli and Waring (1982), such as those with molecular weights 17.5 kDa, 21 kDa, 22 kDa, and 67 kDa. In summary, vitelline membrane proteins are synthesized in follicle cells during the vitellogenic period, and are enriched with proline and alanine.

A gene of a vitelline membrane protein was first cloned in *D. melanogaster* (Higgins *et al.*, 1984). Four cDNAs for the vitelline membrane genes have been cloned and sequenced since then. Thus far, all vitelline membrane genes do not appear to contain any introns. Two genes encoding major vitelline membranes proteins, 17.5 kDa and 23 kDa, are clustered in region 26A of the second chromosome (Burke *et al.*, 1987; Popodi *et al.*, 1988). One gene resides at position 34C (Mindrinos *et al.*, 1985) and another one at 32E (Gigliotti *et al.*, 1989).

Hybrid select translations demonstrate that the vitelline membrane genes indeed code for vitelline membrane proteins (Popodi *et al.*, 1988;

Mindrinos *et al.*, 1985; Gigliotti *et al.*, 1989). The expression of the two genes at 26A locus is limited to the middle and early stages of oogenesis (Popodi *et al.*, 1988). The stage specific expression is also a characteristic of genes from 34C (Mindrinos *et al.*, 1985) and 32E regions (Gigliotti *et al.*, 1989). Follicle cell specific expression of the genes at 26A region is demonstrated by *in situ* hybridization (Burke *et al.*, 1987; Popodi *et al.*, 1988).

Sequence comparison of the *D. melanogaster* vitelline membrane genes revealed a highly conserved region (Popodi *et al.*, 1988; Scherer *et al.*, 1988; Gigliotti *et al.*, 1989). The two genes from 26A (Burke *et al.*, 1987) and 34C regions (Mindrinos *et al.*, 1985) show substantial cross-homology, suggesting considerable similarity in their encoded proteins (Mindrinos *et al.*, 1985). These suggest that the conserved region may be a structural component necessary for the formation of eggshell.

The vitelline membrane gene family and the chorion gene set are evolutionarily related. Their relationship is indicated by cross-hybridization of two vitelline membrane protein genes to the s18-1 chorion protein gene (Higgins *et al.*, 1984; Popodi *et al.*, 1988).

Follicle Cell-Specific Gene

A clone designated as 15A was found by differential screening of the genomic library with cDNA synthesized from Poly A^+ RNA from blood fed female mosquitoes 24 hours post blood meal and from males (Gemmill *et al.*, 1986). A restriction map of this gene was constructed (Fig. 1, Hamblin *et al.*,

1988). *In situ* hybridization using the 350 bp Eco RI-Hind III fragment as a probe to sections of whole mosquito within 50 hours after a blood meal indicated that the gene was expressed in the follicle cells of the ovary, primarily those surrounding the oocyte. For this reason, the gene is also called follicle cell specific (FCS) gene. Northern hybridization demonstrated that 20-hydroxyecdysone was involved in the regulation of FCS gene expression. The gene was expressed in blood fed females, and in blood fed decapitated females after the injection of 20-hydroxyecdysone. The mRNA was detectable at 5 hours post blood meal. Levels were high at 20 to 30 hours, then declined at 40 to 50 hours post blood meal. The localization of the coding sequence was determined to be the 750 bp Eco RI-Xba I fragment (See Fig. 1). Result of S1 nuclease mapping analysis of the FCS RNAs indicated that the 5' end started very shortly upstream of the Eco RI site, and the 3' end of the transcript was 168 nucleotides downstream of the Hind III site (Hamblin *et al*, 1988).

In this report, the FCS gene was studied at the molecular level as a first step towards elucidating the gene product, its characteristics and functions, and the mechanisms controlling its synthesis. The nucleotide sequence of a genomic clone as well as a complete cDNA clone for FCS gene was determined. The amino acid sequence, which includes consensus sites for signal cleavage, polyadenylation signal, and an interesting hydrophobic region, was derived from the analysis of cloned cDNA.

MATERIALS

Chemicals and Equipment

Restriction enzymes, high-temperature-melting and low-temperature-melting agarose, Genius nonradioactive DNA labeling and detection kit, and proteinase K were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). ZAP-cDNA synthesis kit, Uni-ZAP XR vectro kit, and Gigapack II Gold Packiaging Extract were from Stratagene (La Jolla, CA). [α-^{35}S]deoxynucleotide triphosphates were from Amersham (Arlington Heights, IL) or New England Nuclear Research Products (Boston, MA). Nytran membranes for Southern blots were from Schleicher & Schuell (Keene, NH). Absolute ethanol was purchased from Midwest Grain Co. (Pekin, IL). Other reagents were obtained from Fisher Scientific (Pittsburgh, PA) and Sigma Chemical Company (St. Louis, MO). Sequenase kit was from United States Biochemical Corp. (Cleveland, OH). Plasmid DNA preparation kit was from Qiagen (Studio City, CA). X-ray films were Kodak X-OMAT from Eastman Kodak Company (Rochester, NY). Nylon filters were from Gelman Sciences (Ann Arbor, MI). Blue pestles were from Kimble Glass Inc. (Vineland, NJ). Water used for making all the solutions were house-distilled, then passed through a NANOpure II purification system from Barnstead Thermolyne Corp. (Dubuque, IA).

The sequencing apparatus model S2 was from Bethesda Research Laboratories (Gaithersburg, MD). Power supplies included model PS 2500

from Hoeffer Scientific Instruments (San Francisco, CA), and model 200/2.0 from Bio Radiation (Richmond, CA). The gel drier and DNA fluorometer TKO 100 were from Hoeffer Scientific Instruments. The UV detector and camera Foto/Prep I were manufactured by Fotodyne, Inc. (New Berlin, WI). All bacteria and phages were grown using a mechanical convection incubator model 6M from Precision Scientific Inc. (Chicago, IL), and an orbit shaker from Lab-Line (Melrose Park, IL).

Animals

The Rockefeller strain of *Aedes aegypti* (Linnaeus) was employed in all of the experiments. Larval mosquitoes were reared at 26°C under 16 hour light and 8 hour dark. Approximately 200 newly hatched larvae were placed in plastic containers with 450 ml water. Larvae were fed on a diet of ground rat chow, brewer's yeast, and lactalbumin hydrolysate (1:1:1). 1.6 ml of 8% diet solution was provided on day 1, 0.8 ml on day 2, 3.2 ml on day 4, 4.8 ml on day 5, 2.4 ml on days 6 and 7. Pupae were collected on day 8 and then transferred to 1000 ml flasks. Emerged adults climbed to netted cages on paper ladders. Adults were maintained at 26°C and 75% relative humidity, and were provided with 3% sucrose available from cotton wicks. Adult females 3-6 days after eclosion were used for blood-feeding.

METHODS

Nucleotide Sequence Determination

Sequencing template preparation. Sequencing templates were purified using Qiagen plasmid DNA preparation kit. Overnight bacterial cultures were collected, then resuspended in 50 mM Tris-HCl and 10 mM EDTA pH 8.0 with 100 μg/ml RNase A. Bacteria were lysed by adding 200 mM NaOH and 1% SDS. The mixture was neutralized with 2.55 M KAc pH 4.8. Chromosomal DNA, bacterial protein and SDS were precipitated by centrifugation. Then the supernatant was applied to a Qiagen column. Pure plasmid DNA was eluted with buffer containing 50 mM MOPS, 1.2 M NaCl, and 15% ethanol pH 8.0. Template DNA was denatured in 200 mM NaOH solution containing 0.2 mM EDTA at 37°C for 30 min before sequencing reactions.

DNA sequencing. Both restriction enzyme fragments and serial deletion mutants were used for DNA sequencing. Deletion mutants were produced by Dr. Douglas Knipple's laboratory at Geneva, New York. Sequencing was carried out by the dideoxy chain termination method (Sanger *et al.* 1977) using sequenase. Sequencing gel mixture was 5.7% acrylamide, 0.3% N, N'-methylene bisacrylamide, 7 M urea, 100 mM Tris/Borate, and 1 mM Na_2EDTA, pH 8.3, filtered through 0.45 μm nylon filters before use. Gels were allowed to polymerize in the presence of 3 mM TEMED and 1% ammonium persulfate for at least 2 hours, then pre-run for 30-60 min before loading samples. Electrophoresis was performed in Tris/Borate-EDTA buffer pH 8.0 at constant

power of 60 W between 1,700-2,000 volts. Gels were dried with a gel dryer under vacuum at 80°C for 2 hours and then exposed to Kodak X-OMAT x-ray film at room temperature. All sequences presented had been read at least twice on each strand.

Sequence analysis. The sequence data were analyzed using computer programs GCG (Devereux *et al.* ,1984) and MacVector (International Biotechnologies, Inc., New Haven, CT).

cDNA Library Construction and Screening

RNA preparation. The ovaries were hand dissected from mosquitoes 24-30 hours post blood meal in ice cold *Aedes* saline (Hagedorn *et al.*, 1977) and frozen immediately in tubes on dry ice. Total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski & Sacchi, 1987). Ovaries (25) were homogenized with a blue pestle in 600 µl denaturing solution containing 4.2 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, and 0.1 M β-mercaptoethanol. 60 µl sodium acetate pH 4 was first added into the homogenate. Then 600 µl water saturated phenol and 120 µl chloroform/isoamyl alcohol (49:1) were also added. The RNA was then precipitated with isopropanol, washed with 75% ethanol, and dried under vacuum. Precipitated RNA was dissolved in sterile water, and was stored at -80°C.

cDNA library construction. A cDNA library was constructed using the Uni-ZAP cDNA cloning system from Stratagene with total RNA prepared from

170 ovaries. The first strand cDNA was converted from total RNA and oligo-dT primer in conjunction with an Xho I recognition sequence by moloney-murine leukemia virus reverse transcriptase (M-MuLVRT) in the presence of dATP, dGTP, dTTP and 5-methyl dCTP. The second strand cDNA was synthesized using RNaseH and *Escherichia coli* DNA polymerase I. The resulting double-stranded cDNA was blunted with T4 DNA polymerase. Eco RI adaptors were attached to both ends by adding T4 DNA ligase. The Xho I enzyme released the Xho I recognition site at the 3' end for directional cloning. Free linkers were removed by applying the reaction mixture to a Sepharose CL-4B column. The DNA was packaged with Gigapack II Gold packaging extract after the double-stranded cDNA was ligated to the λ-ZAP II vector. The library was subsequently plated on the *E. coli* cell line PLK-F', and was amplified immediately. The suspension in SM buffer, which contained 5.9 g NaCl, 2 g MgSO4, 50 ml 1M Tris-HCl pH7.5, and 5 ml 2% gelatin in one liter solution, was added with chloroform to 0.3%. The amplified library was stored in aliquots at 4^oC.

cDNA library screening. The cDNA library was screened by *in situ* plaque hybridization. The nucleic acids were crosslinked to the filter by ultraviolet irradiation. The 0.35 kb Eco RI-Hind III fragment from the genomic clone was ^{32}P-labeled by random primer labelling according to manufacture's instruction (Bethesda Research Laboratories). The unincorporated nucleotides were removed from the probe reaction mixture using a Sepharose CL-4B column. The hybridization and washing were carried out under high stringency conditions (Sambrook *et al.*, 1989). The membranes were prehybridized in 5xSSC, 0.1% SDS, 0.1% NaHPO4, 1xDenharts, and 100

μg/ml fish sperm DNA at 65°C for 2 hours. Using fresh prehybridization solution, the membranes were hybridized with $1x10^5$ cpm/ml of probe at 65°C for 18 hours. The hybridized membranes were washed twice in 2xSSC, 0.1% SDS at room temperature for 5 minutes each. This wash solution was replaced with 0.2xSSC, 0.1% SDS and washed twice for 30 minutes each at 65°C. After washing, the membranes were air dried and exposed overnight to Kodak X-OMAT film with a Dupont Lightening Plus intensifying screen at -70°C.

In vivo excision of pBluescript. The selected Uni-ZAP XR clones were converted to pBluescript SK^- phagemid by superinfection with a filamentous helper phage R408.

Genomic Southern Analysis

Probe preparation. Eco RI and Hind III restriction fragments were isolated from FCS genomic clone Plasmid DNA of genomic clone was digested with restriction enzymes Eco RI and Hind III. Digested DNA was fractionated in 1% high melting agarose gel in 1xTAE, *i.e.*, 40 mM Tris/Acetate and 2 mM EDTA pH 8.5. A 5 mm section of the gel was cut out in front of the desired band after staining and visualizing. The gap was filled with a molten solution of 0.7% low melting agarose. The desired DNA fragment was electrophoresed into the low melting agarose after solidification, and then was excised. The gel slice was melted at 65°C for 5 min. NaCl was added to a final

concentration of 8%. Finally the DNA was purified by phenol/chloroform extraction, followed by ethanol precipitation.

Genomic DNA preparation. A total of 80 to 100 mosquito pupae which molted from the fourth instar larvae were collected within 4 hours, washed with diluted bleach (0.87% sodium hypochlorite) and then with distilled water. Animals were ground in liquid nitrogen in a motar until a fine powder was obtained. This grindate was homogenized in a Dounce homogenizer in 3 ml buffer containing 80 mM EDTA and 100 mM Tris-HCl pH 8.0, plus 160 mM sucrose. SDS and proteinase K were then added to final concentrations of 0.5% and 0.17 mg/ml, respectively, and the mixture was incubated at 70°C for 1 hour. Subsequently, nucleic acids were extracted sequentially using phenol saturated with 100 mM Tris pH 8.0, phenol/chloroform/isoamyl alcohol [24:24:1 (vol/vol)], and chloroform. Nucleic acids were precipitated by first adding KAc to a final concentration of 1.2 M and then adding an equal volume of ethanol. RNAs were digested by adding DNase-free RNase to 0.025 mg/ml and incubated at 60°C for 20 min. Total DNAs were then collected by precipitating with 0.3 M NaAc and two times volume of ethanol. The precipitates were washed with 70% ethanol, dried under vacuum, and resuspended in 10 mM Tris-HCl and 1 mM EDTA pH 8.0. DNA concentrations were determined using a DNA fluorometer.

Genomic blotting and hybridization conditions. Restriction analysis employed six different enzymes: Bgl II, Eco RI, Hind III, Kpn I, Sac I, and Xba I. Each reaction mixture contained 10 µg of genomic DNA and one of the restriction enzyme buffer systems recommended by the manufacturer. Fifty units of

enzyme and 5 mM spermidine were added, and the reactions were incubated at 37°C for 6 hours. Thereafter, digested DNAs were fractionated by electrophoresis at a constant voltage of 60 V at room temperature for approximately 14 hours. Gels containing genomic DNA were denatured with 0.5 N NaOH and 1.5 M NaCl, neutralized with 1 M Tris pH 8.0 and 1.5 M NaCl, then transferred onto Nytran membranes in 20xSSC (1xSSC: 0.15 M NaCl/0.015 M Sodium citrate pH 7.0) overnight. Afterwards, DNAs were blotted to the membranes by incubating at 80°C for 2 hours in an oven. The membranes were prehybridized in 5xSSC, 0.1% Sarkosyl, 1% SDS, 5% blocking reagent, and 50% formamide at 42°C. Subsequently, hybridizations were performed in the same condition supplemented with 50 ng/ml Dig-dUTP labeled probes. Membranes were washed successively in 2xSSC and 0.1% SDS at room temperature, then in 0.1xSSC and 0.1% SDS at 68°C for 30 min. Genomic blots were submerged in blocking solution containing 1% blocking reagent and 50 µg/ml salmon sperm DNA. Membranes were incubated with 150 mU/ml antibody-conjugate, *i.e.*, anti-digoxigenin-alkaline phosphatase, to detect the labeled probes. Desired bands were visualized using X-phosphate and nitroblue tetrazolium (NBT) salt.

Plasmid DNA preparation. All plasmid DNAs were prepared using the method described by Alter and Subramanian (1989). 1.5 ml overnight culture was collected by centrifugation and resuspended in 10 mM Tris and 1 mM EDTA pH 8.0, plus 1.25 M NaCl. Thereafter, cells were lysed, and proteins were removed by phenol/chloroform which was pre-equilibrated with 1 M Tris pH 7.8 and 1 M NaCl. Chromosomal DNA was then precipitated by the NaCl

in the phenol. The aqueous phase containing plasmid DNA was collected and the plasmid DNA was precipitated by ethanol.

Restriction analysis for plasmid DNA. Restriction endonuclease digestions of DNAs were performed as suggested by the supplier (Boehringer Mannheim Biochemicals). The excess of RNA was removed by a brief incubation with RNase A prior to electrophoresis of the restriction-digested samples.

RESULTS

Organization of the Follicle Cell-Specific Gene

In order to study the transcriptional regulation of the FCS gene, it was determined to characterize the gene in detail and to define the upstream sequence. Based on the restriction map (Fig. 1) constructed by Hamblin *et al.* (1988), the 1.5 kb Xba I-Xba I fragment was chosen to produce serial deletion mutants for sequencing by Dr. Douglas Knipple (Cornell University). The fragment being selected was believed to contain about 750 bp of upstream sequence, 600 bp of coding sequence, and 150 bp of downstream sequence. The sequencing began with the whole set of serial deletion mutants from Dr. Knipple in the plasmid pBluscript II SK$^-$, two of the 1.5 kb Eco RI-Eco RI inserts and the 0.35 kb Eco RI-Hind III insert in the plasmid BBM$^-$.

Unfortunately, results obtained from the DNA sequence did not agree with the restriction map provided by Hamblin *et al.* (1988). Fig. 2 shows the revised organization of the genomic clone and the strategy used to sequence the gene. Particularly, the coding sequence Eco RI-Hind III fragment was at the 5' end of the clone. Thus, the genomic clone did not contain the upstream sequence of the gene. The focus of the project was therefore changed toward obtaining a cDNA clone of this gene.

Isolation of Follicle Cell-Specific Clones from Ovarian cDNA Library

A cDNA library was constructed with total RNA from mosquito ovaries using the Stratagene ZAP-cDNA synthesis kit and the Uni-ZAP XR vector kit. A library containing 35,000 recombinant phages was obtained. Then the library was amplified to 44 ml with $3x10^5$ clones per µl, *i.e.*, $1.3x10^{10}$ clones in total. About 9,000 clones were screened by *in situ* plaque hybridization, and approximately 80 positive clones were obtained. Twenty of them giving strong signals were used for secondary screening. Twelve positive clones were used to conduct *in vivo* excision to rescue phagemid SK^-. Seven of them were selected to isolate plasmid DNA. Restriction analysis showed that they all contained about 550 base pair inserts with the Eco RI-Hind III fragment. Finally, the DNAs from all seven selected clones were sequenced. The sequence of Eco RI-Hind III fragment from the cDNA clones and that from the genomic clone were virtually identical. This suggested that the longest clone, 3E2a containing 580 base pair insert, was the full length one since its size and restriction map were matched with the transcribed region that Hamblin *et al.* (1988) predicted from the genomic clone (Fig. 2). Fig. 3 presents the organization of the cDNA clone. The direction of transcription was established using the cDNA clone. The entire nucleotide sequence is shown in Fig. 4.

General Features of the Follicle Cell-Specific Gene

The nucleotide sequence of the cDNA contained 571 bp, including 57 bp 5'-noncoding region, 324 bp coding frame, 190 bp 3' untranslated region,

and 18 bp poly (A) stretch (Fig. 4). The reading frame for this sequence was determined from the fact that only one frame contained no termination codons. The other two frames contained 3-8 terminators, generally distributed over much of the sequence. The open reading frame began with ATG in a context that followed the most favorable sequence for translational initiation proposed for *Drosophila* genes (C/AAAC/A, Cavener, 1987) and general eukaryotic genes (A/GCCATGG, Kozak, 1986). An in-frame TAA stop codon appeared at 382 bp. A hexamer, AATAAA, corresponding to the polyadenylation signal (Proudfoot & Brownlee, 1976; Zarkower & Wickens, 1987) ocurred at position 534 in the 3' untranslated region. The C at 553 bp was the most likely polyadenylation site. A consensus sequence YGTGTTYY, which was proposed to be required for the efficient formation of correct mRNA 3'-termini in transcription (McLauchlan *et al.*, 1985), was found at 514 bp.

Sequence Analysis and Comparison

The cDNA of the FCS gene encoded a protein precursor of 108 amino acids with a predicted relative molecular mass of 9.5 kDa. Hydropathy analysis (Fig. 5) showed that the first 18 amino acids of the peptide fragment had high hydrophobicity. This amino acid sequence was similar to the signal peptides of many other export éukaryotic proteins. Namely, they all shared the "positive-hydrophobic-polar" feature (von Heijne, 1990). The cleavage site for the signal sequence was predicted according to von Heijne (1983 and 1984). The first 18 amino acid sequence seemed to be a signal peptide for translocation of the newly synthesized protein across a membrane.

Nevertheless, the prediction that the end of the signal sequence was at amino acid 18 (Fig. 4) remained speculative, since the N-terminal amino acid sequence of the mature protein was not known.

The cDNA deduced peptide sequence was used to search the NBRF protein data base (release 25.0) using the FastA program (Pearson and Lipman, 1988). It revealed that the hypothetical FCS peptide and the *Drosophila* vitelline membrane proteins had high similarity (Fig. 6).

Southern Blot Analysis of Genomic DNA

The number of the follicle cell-specific genes present in the *A. aegypti* genome was investigated. The strategy was to hybridize a labeled gene fragment to electrophoretically separated restriction fragments of total *A. aegypti* DNA. The number of genes was estimated by a comparison of the hybridization signals from different restriction digestions of total DNA. In this experiment, DNA from primarily diploid cells of pupal stage mosquitoes was digested with different restriction enzymes: Bgl II, Eco RI, Hind III, Kpn I, Sac I, and Xba I, and then electrophoresed. 10 μg of mosquito DNA was loaded on each lane. Then a Southern blot of this gel was hybridized to a digoxigenin-labeled Eco RI-Hind III fragment isolated from the genomic clone. Visualization of the filter showed a single strong band appearing in the digestions of Bgl II, Eco RI, Hind III, Kpn I, Sac I, and Xba I, with sizes of 9 kb, 1.5 kb, 2 kb, 12 kb, 1.8 kb, and 3.2 kb, respectively (Fig. 7). Since no other signal with similar

intensity ocurred in any lane, it appeared that only one copy of the FCS gene was present in the *A. aegypti* genome.

A Gene Similar to the Follicle Cell-Specific Gene

A false-positive clone, 1D, was isolated during the cDNA library screening. The cDNA having 486 bp was then sequenced (Fig. 8). Many CC repeats appeared in the nucleotide sequence, which was similar to the FCS gene. This cDNA and the cDNA of FCS gene had 60% similiarity at the nucleotide level (Fig. 9). The longest reading frame of this gene lead to a translation product having multiple proline residues. A short sequence Asp-Tyr-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro (DYPAPPPPPP) in this reading frame was very similar to a decapeptide found in the *A. aegypti* ovary by Borovsky *et al.* (1990). The peptide isolated by Borovsky *et al.*, YDPAPPPPPP, was reported to be able to inhibit trypsin activity by inhibiting biosynthesis of trypsin-like enzyme. This reading frame of the clone 1D would produce a peptide of 92 residues. However, no in-frame ATG was in this reading frame. Therefore, this clone may not be of full length.

A nucleotide sequence, TTTTTAT, appeared at position 402 in the 3' untranslated region of clone 1D. This sequence was demonstrated to be important for progesterone-induced mRNA maturation in the frog oocyte (Fox *et al.*, 1989). When the 1D sequence was used to search the NBRF protein data base using the FastA program (Pearson and Lipman, 1988), a *Drosophila* vitelline membrane protein 34C (Mindrinos *et al.*, 1985) also

appeared to be highly similar to the deduced peptide of 1D with 38% identity (Fig. 10).

DISCUSSION

Coding Potential of the Follicle Cell-Specific Gene

The genomic sequence of the FCS gene from *A. aegypti* was determined using both restriction fragments and serial deletion mutants. A cDNA clone corresponding to the genomic clone was characterized at the nucleotide level. A hypothetical protein was derived from the cDNA sequence as a precursor with an 18 amino acid signal peptide. The mature protein had 90 amino acids, and had a molecular weight of 9.5 kDa. By searching protein data base NBRF, a vitelline membrane protein from *D. melanogaster* (Mindrinos *et al.*, 1985) exhibited approximately 36% identity to the translated peptide at the amino acid level.

The FCS gene from *A. aegypti* and the vitelline membrane protein genes from *D. melanogaster* (Burker *et al.*, 1987; Gigliotti *et al.*, 1989; Higgins *et al.*, 1984; Popodi *et al.*, 1988) are remarkably similar in their sex, tissue, and stage specific expressions. In *A. aegypti,* the FCS gene transcript is detectable between 5 to 50 hours after blood feeding, and is especially high at 20-30 hours. The gene is expressed in the perioocyte follicle cells (Hamblin *et al.*, 1988). Likewise, the vitelline membrane proteins produced in the follicle cells of *D. melanogaster* are synthesized and deposited during the vitellogenic period (Fargnoli and Waring, 1982). In short, the genes in both animals are expressed in the vitellogenic stage, specifically in the follicle cells of the ovary.

In *A. aegypti,* the control of the FCS gene by 20-hydroxyecdysone (Hamblin *et al.*, 1988) is apparently unique for vitelline membrane protein. In comparison, no direct hormonal control on eggshell formation has been demonstrated so far in *Drosophila*. Orr *et al.* (1989), however, suggest that ecdysone could be involved in the regulation of choriogenesis in *Drosophila*. They have shown that the *de 12* mutation, which results in clearly abnormal chorionic appendages, is allelic to *l(1)npr-1*. The *l(1)npr-1* gene is part of the *Broad-Complex* locus residing at the 2B5 salivary gland "early" puff, and appears to play an important role in the mediation of ecdysone effects (Meyerowitz *et al.*, 1985). This locus is necessary for various chromosomal puffs to expand and regress properly during late larval development, as well as for certain ecdysone-dependent gene to be expressed. The discovery of a steroid hormone receptor-like protein and a proposed steroid hormone receptor binding DNA element in a chorion gene supports the hypothesis of steroid hormone involvement of choriogenesis (Shea *et al.*, 1990).

A comparison of FCS DNA with *D. melanogaster* vitelline membrane gene 26A (Burke *et al.*, 1987) reveals that their deduced translation products have 46.7% similarity at the amino acid level (Fig. 6). Between amino acid position 54 to 88 of FCS and between 73 to 108 of 26A, the sequences are best conserved, showing 60% similarity. In this region, 14/35 or 40% of the residues are identical (excluding a gap of 1 residue). The deduced signal peptides of FCS and 26A are similar by 72%. In both genes, the 5' untranslated regions are similar in nucleotide sequence by approximately 45%. The overall nucleotide similarity in the coding region between FCS and 26A is 47.5%. Clearly, the FCS and 26A sequences are related.

Interestingly, comparison of the protein encoded by the FCS gene from *A. aegypti* with those published vitelline membrane proteins from *D. melanogaster* reveals conserved hydrophobic regions among the coding region of these peptides (Fig. 11). Conservation of the hydrophobic region in different proteins may be significant for protein structure. This suggests that the hydrophobic region is a structural component necessary for the formation of eggshells. The vitelline membrane is deposited before the wax layer and the chorion are formed. Studies in the egg formation of other insects have suggested that the vitelline membrane is comprised of a set of overlapping plates (Papanikolaou *et al.*, 1986), which helps the oocyte to resist osmotic shrinkage at the early stage of egg formation (Telfer and Smith, 1971). Therefore, it is possible that the hydrophobic region is important for such a function.

The Vitelline Membrane

In *D. melanogaster*, the vitelline membrane is a very thin noncellular layer outside of the oocyte plasma membrane, and beneath the chorion (Margaritis *et al*, 1980). Vitelline membrane materials, known as vitelline bodies, are secreted during stage 9 of egg development, fuse subsequently at stage 10 forming a 1.7 μm thick membrane which gradually thins to 0.4 μm as oogenesis proceeds. The vitelline membrane is highly elastic. Regional modification of the vitelline membrane occurs in the hatching region, which has two discontinuous endochorionic halves kept together by a common layer of fragile inner endochorion and a layer of exochorion. This area of weakness

facilitates the escape of the larvae (Turner and Mahowald, 1976). Vitelline membrane regional protrusion forms the micropyle together with the chorion. Its endochorionic component is an open-ended and hollow cone, into which fits a protuberance of the vitelline membrane. The micropyle is 0.7 μm in diameter and 7 μm in length. This is just sufficient to allow the 1.7 mm long and 0.6 μm wide sperm to enter the oocyte (Zarani and Margaritis, 1986). Polyspermy may thus be restricted by the physical dimensions of this structure (Perotti, 1974).

In *A. aegypti,* vitelline membrane plaques begin to appear between the follicular epithelium and the oocyte approximately 24 hours after a blood meal (Anderson and Spielman, 1971), which is during the peak of yolk protein uptake by micropinocytosis. The plaques become larger and closely packed about 30 hours post blood meal. The vitelline plaques then fuse together forming a continuously homogeneous layer approximately 1.2 μm thick by 40 hours post blood meal, by which time vitellogenin uptake ceases. The endochorion consists of two layers. The lower layer is dense homogeneous pillar-like structures alternating with fibrous mesh-like areas, and the outer layer is fibrous. Deposition of the endochorion is completed about 48 hours post blood meal and the follicle cells then degenerate (Mathew and Rai, 1975; Powell *et al.*, 1988).

Amino Acid Analysis

All insect eggshell layers except the wax layer are composed mainly of proteins, with lipids and carbohydrates also being present. The protein encoded by FCS gene has not been purified or characterized yet. However, analysis shows that the deduced translation product is generally similar to the vitelline membrane proteins of *Drosophila* (Table 1) and is also rich in proline and alanine.

Since the protein compositions of FCS and *Drosophila* vitelline membrane are distinct in nature from any other proteins, it is informative to compare the amino acid compositions of FCS with those of *Drosophila* vitelline membranes. The amino acid compositions of their translation products are calculated from the nucleotide sequences of FCS, 26A (Burke *et al.,* 1987), 32E (Gigliotti *et al.,* 1989), 34C (Mindrinos *et al.,* 1985), and Tu4 (Popodi *et al.,* 1988). The similarities between the amino acid composition of the FCS and *Drosophila* vitelline membrane proteins are shown in Table 1. They are both low in methionine and high in alanine and proline.

Amino acid analysis of protein encoded by the FCS gene reveales some general similarities to eggshell proteins such as class C chorion proteins in *A. polyphemus* (Table 2). All of these proteins have a high percentage of nonpolar amino acids (54%), an especially high content of glycine and alanine, and a low content of charged amino acids (24.3%). However, relative to proteins of class C chorion, FCS is depleted in methionine and glycine and rich in proline. Tandem Gly-Tyr-Gly-Gly-Leu

related repeats are characteristic of the chorion protein. Such repeats are absent from the FCS. The FCS gene protein shows additional differences for amino acids such as lysine, threonine, histidine and multiple proline residues.

The predicted vitelline membrane protein from *A. aegypti* has a higher histidine content than the proteins from *D. melanogaster* (Table 1). However, this does not rule out the possibility that FCS gene product is a vitelline membrane protein. High histidine composition in the vitelline membrane has been previously observed in *A. aegypti.* By injecting ^{3}H-histidine into the mosquitoes after blood-feeding, histidine is found to be incorporated into follicle cells, and later the vitelline membrane plaques, secreted from the follicle cell, are heavily labelled (Anderson and Spielman, 1973).

Mosquito follicle cells are highly specialized for synthesizing a special set of proteins. Among these are the chorion and proteins, neither of which have been examined in the mosquito. Given the convenient size and hormonal control of expression, the vitelline membrane gene will provide a convenient model for examining the developmental and hormone regulated expression of genes important in mosquito reproduction. Expression of the gene *in vitro* will allow characterization and investigation of the functional roles of the protein.

Nature and Significance of 1D

Sequence comparison showed that the genes FCS and 1D were highly homologous. They had 60% similarity at nucleotide level, and had 79.8%

similarity at the amino acid level with 67.9% identity. The matrix analyses are shown in Fig. 12 and Fig. 13, respectively. A hydrophobic region was also presented in the 1D translation product between 60 to 80 residues (Fig. 14) which showed 90% similarity and 83% identity at the amino acid level with the FCS gene (Fig. 11). This suggested that these two genes may have common evolutionary origin, and that they are both members of a family of vitelline membrane proteins.

Similar to the hypothetical FCS peptide, the 1D translation product showed high content of alanine (18.5%) and proline (30%), low in methionine (0) and exceptionally high histidine (10.9%).

Similarity between the 1D hypothetical peptide and the decapeptide isolated by Borovsky *et al.* (1990) raised some interesting questions. Both peptides were from the ovaries of blood fed *A. aegypti*, and their sequences were identical except that the first two amino acids were reversed. The 1D gene could be a non-translated gene related to the gene coding for the decapeptide isolated by Borovsky *et al.*. Alternatively, the peptide isolated by Borovsky *et al.* could be a degradation product of a vitelline membrane protein. In the 1D gene a lysine immediately followed a string of 6 prolines, providing a possible site for cleavage. The alanine immediately before the asparagine of the decapeptide was a convenient cleavage site for a possible signal peptide. Thus it is possible that the peptide purified by Borovsky *et al.* is not a hormone, but a degradation product. The requirement for millimolar concentrations for activity (Borovsky *et al.*, 1990) suggests that it is not a true hormone.

Direct *in vivo* and *in vitro* functional assays will be required to document the importance of 1D in mosquito egg development. Availability of the cDNA clone will allow *in vitro* expression of 1D protein fused to a strong promoter. If purification of the 1D protein is possible, antibody can also be produced. Therefore, the native protein from the mosquitoes can be identified, and the physiological functions of this protein can be investigated. It would be interesting to see if the decapeptide isolated by Borovsky *et al.* (1990) could be prepared from the 1D protein. Assuming another gene coding for the decapeptide with the correct sequence can be isolated and expressed, one may be able to develop tools needed to ask relevant physiological questions: Is the decapeptide normally produced from the translation product and is it found in the hemolymph?

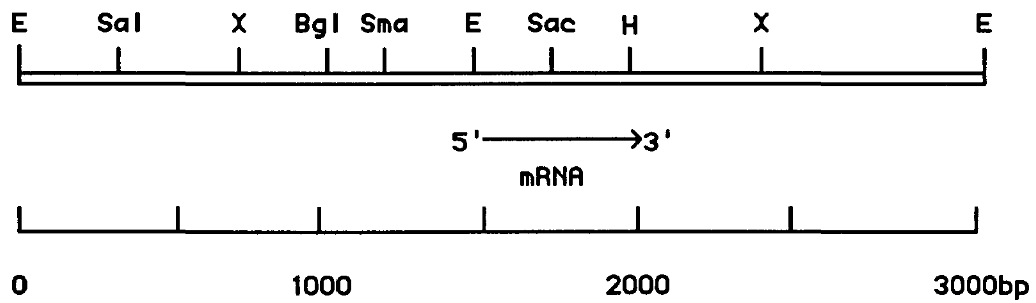

FIG. 1. Original restriction map of the FCS genomic clone constructed by Hamblin *et al.* (1988). Restriction endonuclease designations are as follows: E, Eco RI; H, Hind III; X, Xba I; Sac, Sac I; Sma, Sma I; Bg, Bgl II; Sal, Sal I.

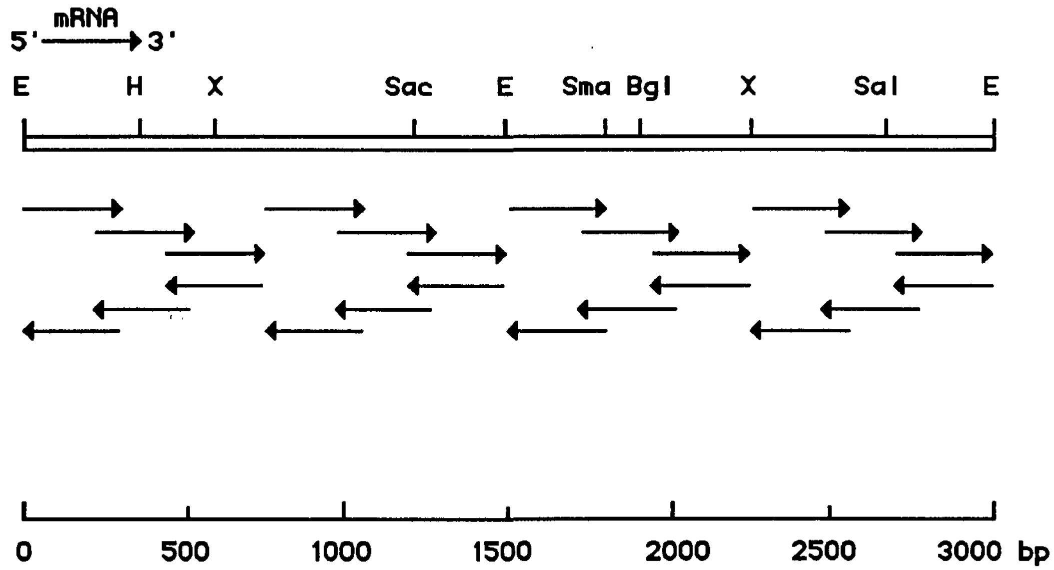

FIG. 2. Corrected restriction map of the FCS genomic clone and sequencing strategy. Arrow above the DNA strands indicates the direction of transcription. Arrows below the DNA strands indicate directions in which sequences were read. Restriction endonuclease designations are as follows: E, Eco RI; H, Hind III; X, Xba I; Sac, Sac I; Sma, Sma I; Bg, Bgl II; Sal, Sal I.

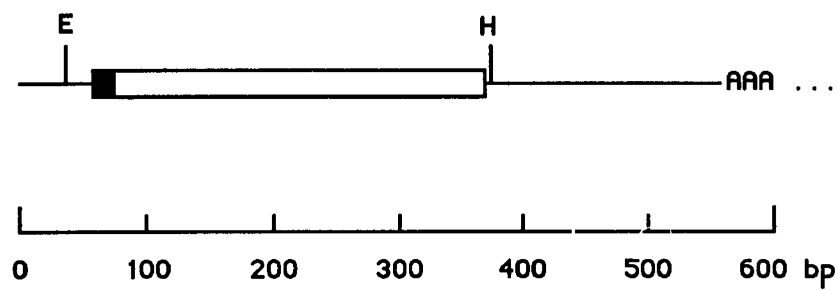

FIG. 3. Structure of the FCS cDNA clone. Closed and open rectangles represent, respectively, the predicted signal sequence and the mature protein. Restriction endonuclease designations are as follows: E, Eco RI; H, Hind III. AAA... indicates the poly(A).

```
1      ACTTGCCCAACAGTGTACTACAGTCCCACCGAGGAATTCCCATCCAACTCAGTAACC   57

58   ATG AAC AAG TTC ATC ATC TTG GCA ATC TTC GCT CTG GCC GTC GGC   102
1    Met Asn Lys Phe Ile Ile Leu Ala Ile Phe Ala Leu Ala Val Gly   15

103  GCC ATG GCT GAT TAC CCG AAG CCA GCT TAC CAC GCA CCT CCT CCA   147
16   Ala Met Ala Asp Tyr Pro Lys Pro Ala Tyr His Ala Pro Pro Pro   30

148  CCA CCA CCA CAC CAC CTC CAC GCT CAC CCC GCT CCG GCT CCA GTG   192
31   Pro Pro Pro His His Leu His Ala His Pro Ala Pro Ala Pro Val   45

193  GTT CAC ACC TAC CCG GTG CAC GCC CCC CAC GCC AAG TGC GGT GCC   237
46   Val His Thr Tyr Pro Val His Ala Pro His Ala Lys Cys Gly Ala   60

238  AAC CTG CTG GTA GGA TGC GCC CCA AGC GTT GCC CAC GTC CCA TGT   282
61   Asn Leu Leu Val Gly Cys Ala Pro Ser Val Ala His Val Pro Cys   75

283  GTC CCC TTG CCA GGA CAT GCT CCC GCT CAC GGA TAC GGA CAC GCT   327
76   Val Pro Leu Pro Gly His Ala Pro Ala His Gly Tyr Gly His Ala   90

328  CCA GCC CCA CAC TAC CGT GCC CCG GAA TCG GAC TCG TTC GAC CAG   372
91   Pro Ala Pro His Tyr Arg Ala Pro Glu Ser Asp Ser Phe Asp Gln   105

373  TTC GAG GAATAAGCTTCCGGTCCCACTTGAAGGCTCTTGAAGATGCAGCGGAAAATA   429
106  Phe Glu Glu

430  CTCGACACGGCAATGTCCAAATTAATTATGAGACACGGAACGCGATCAGGATTTCGGAA   488

489  ATGGTAGCAAAAGTGTGACTAATACTGTGTGTTGCGCAGCGCATGAATAAATTGCTATT   547

548  TAAAACAAAAAAAAAAAAAAAAAA  571
```

FIG. 4. The nucleotide sequence of a FCS cDNA and deduced amino acid sequence. Nucleotides are numbered in the 5' to 3' direction beginning with the first nucleotide of the cDNA. Amino acids are numbered from the initiation methionine. The amino acids 1-18 marked with dots are presumed to be the signal peptide sequence. A hydrophobic region (amino acids 54-88) is boxed. The underlined sequence is presumed to be the poly (A) signal. The YGTGTTYY element is double underlined.

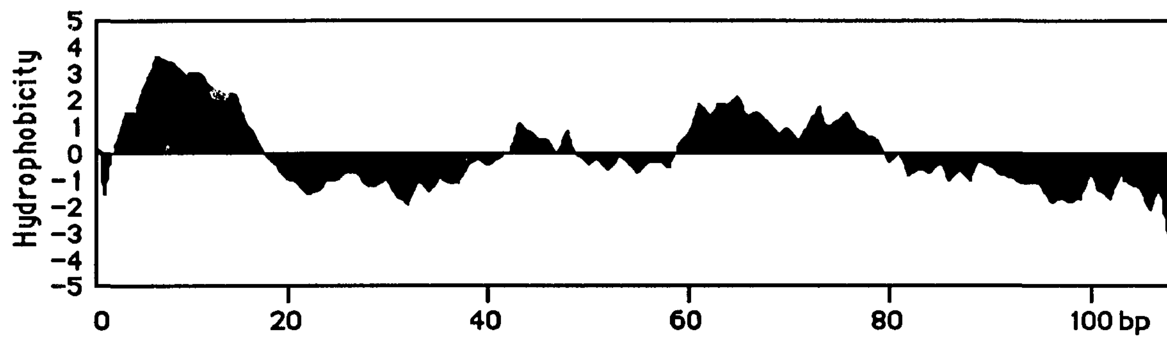

FIG. 5. Hydropathy plot analysis of the deduced FCS peptide sequence according to the method of Kyte and Doolittle (1982).

```
FCS    1 MNKFIILAIFALAVGAMADYPKPAYHAPPPPPPHHLHAHP  40
26A    1 MKSFVCIALVAFAAAALASPTNVASATGSTGSSVTTQDGE  40

FCS   41 APAPVVHTYPVHA...................PHAKCGAN  61
26A   41 LEGVTGQGFGDLTRLRKSAYGGSSGGYGGSSIPAPPCPKN  80

FCS   62 LLVGCAPSVAHVPC.VPLPGHAPAHGYGHAPAPHYRAPES 100
26A   81 YLFSCQPNLAPVPCSAPAPSYGSAGAYS.SPVATYVAPNY 119

FCS  101 DSFDQFEE.............. 108
26A  120 GVPQHQQQLYSAYVPQTYGYQY 141
```

FIG. 6. Comparison of the translated peptide sequences from the FCS (*A. aegypti*) and 26A (*D. melanogaster*) using the Gap program in the GCG package. Identical residues are enclosed in boxes. Residues whose comparison value is greater than or equal to 0.50 are indicated by ":" and are in bold type letters. Residues whose comparison value is greater than or equal to 0.10 are indicated by ".". Gaps which improve sequence alignment are shown by dots.

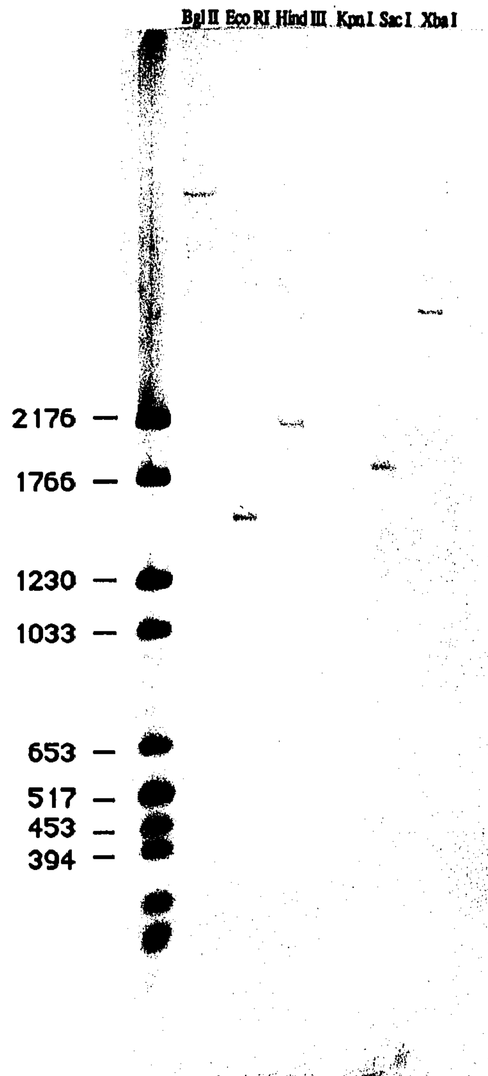

FIG. 7. Genomic Southern analysis of the FCS gene in the *Aedes aegypti* genome.

```
  1  GCA GCA TTG GTC CTC TTC ACC GCC GTC ATC GGT GCC CTG GCT GAC   45
  1  Ala Ala Leu Val Leu Phe Thr Ala Val Ile Gly Ala Leu Ala Asp   15

 46  TAT CCA GCC CCA CCT CCA CCA CCA CCG AAG CCA TAC CAC GCC CCT   90
 16  Tyr Pro Ala Pro Pro Pro Pro Pro Pro Lys Pro Tyr His Ala Pro   30

 91  CCC CCA CCA CCG TAC CAC GCC CCA CCC CAC CAT GCT CCG GCC CCA  135
 31  Pro Pro Pro Pro Tyr His Ala Pro Pro His His Ala Pro Ala Pro   45

136  CTC CAC CCC GTT GTC CAC ACA TAC CCG GTC AAG GCC CCT GCT GCC  180
 46  Leu His Pro Val Val His Thr Tyr Pro Val Lys Ala Pro Ala Ala   60

181  AAG TGC GGT GCT AAC CTT CTG GTA GGA TGC GCT CCA AGC GTT GCC  225
 61  Lys Cys Gly Ala Asn Leu Leu Val Gly Cys Ala Pro Ser Val Ala   75

226  CAC GTG CCA TGC GTC CCG GTG CAC CCA CAC CCA CCA CCA CCA GCC  270
 76  His Val Pro Cys Val Pro Val His Pro His Pro Pro Pro Pro Ala   90

271  CAC TAC TAAGCGGTGACCAGACCACCCAACCCGATGACGGCGGACAAGGCCTCGGAA  327
 91  His Tyr

328  CCAGGACCAAGGAACGGTACAACGATGCGGCACAGTTGATGCGGAATAGTATTAAACAA  386

387  ATTGTTTTATGTTTCTTTTTATTTGGAGGATTTGGATTGAATGTTGTGAGTGCGAAGGA  445

446  GAATAAATTGTTAGTTTTTCTATAAAAAAAAAAAAAAAAAA  486
```

FIG. 8. DNA sequence of the coding strand and conceptual translation of the 1D gene. The amino acids 1-14 marked with dots are presumed to be the signal peptide sequence. The DYPAPPPPPP sequence is bold underlined. The putative hydrophobic domain is boxed. The TTTTTAT sequence is waved underlined. A polyadenylation signal, AATAAA, is underlined in the 3'-untranslated region.

```
FCS   1 MNKFIILAIFALAVGAMADYPKPAYHAP.....PPPPPHH  35
              |.:|. .:||:|||| |: .:|     |||||.|
1D    1 ....AALVLFTAVIGALADYPAPPPPPPKPYHAPPPPPYH  36

FCS  36 LHAHPAPA...PVVHTYPVHAPHAKCGANLLVGCAPSVAH  72
         .:|.|||   ||||||||.|| |||||||||||||||||
1D   37 APPHHAPAPLHPVVHTYPVKAPAAKCGANLLVGCAPSVAH  76

FCS  73 VPCVPLPGHAPAHGYGHAPAPHYRAPESDSFDQFEE  108
        |||||:..|:|       |::||
1D   77 VPCVPVHPHPP.......PPAHY.............  92
```

FIG. 9. Optimal alignment of the FCS and 1D genes. The two sequences start "out of phase" (terminal gap). Identical residues are boxed. Residues whose comparison value is greater than or equal to 0.50 are indicated by ":" and are in bold type letters. Residues whose comparison value is greater than or equal to 0.10 are indicated by ".". Gaps which improve sequence alignment are shown by dots.

```
1D    1 ...............AALVLFTAVIGA...........LA 14
                       ..|.|:.|.::|           :
34C   1 SGNHQPINMKCIAIVSTLCLLAAFVAADKEDKMLGSSYGG 40

1D   15 DYPAPPPPPPKPYHAPPPPPYHAPPHHAPAPLHPVVHTYP 54
        :|. |:::|:..| ||:::   .|. :|||:  |   . |
34C  41 GYGKPAAAPAPSYSAPAAA...SPGLRAPAA..PSYAAAP 75

1D   55 VKAPAAKCGANLLVGCAPSVAHVPCVPVHPHPPPPAHY 92
        |. ||:.|. |.|.:|.|.:|.||| :.:
34C  76 VSIPAPPCPKNYLFSCQPNLAPVPCSAPR......... 104
```

FIG. 10. Comparison of the translated peptide sequences from the 1D (*A. aegypti*) and 34C (*D. melanogaster*). Identical residues are enclosed in boxes. Residues whose comparison value is greater than or equal to 0.50 are indicated by ":" and are in bold type letters. Residues whose comparison value is greater than or equal to 0.10 are indicated by ".". Gaps which improve sequence alignment are shown by dots.

A. aegypti	FCS	C	G	A	N	L	L	V	G	C	A	P	S	V	A	H	V	P	C
A. aegypti	1D	C	G	A	N	L	L	V	G	C	A	P	S	V	A	H	V	P	C
D. melanog	26A	C	P	K	N	Y	L	F	S	C	Q	P	N	L	A	P	V	P	C
D. melanog	34C	C	P	K	N	Y	L	F	S	C	Q	P	N	L	A	P	V	P	C
D. melanog	32E	C	P	T	N	Y	L	F	S	C	Q	P	N	L	A	P	A	P	C
D. melanog	Tu4	C	P	K	N	Y	L	F	S	C	Q	P	S	L	Q	P	V	P	L

FIG. 11. Amino acid sequences of the hydrophobic region in the predicted FCS and 1D gene products with vitelline membrane proteins previously reported from *Drosophila melanogaster.* Conserved amino acids in all six peptides are boxed with solid line. Dotted boxes enclose identical amino acids that are found in two or more sequences. Conserved cysteines between sequences are contiguous from line to line.

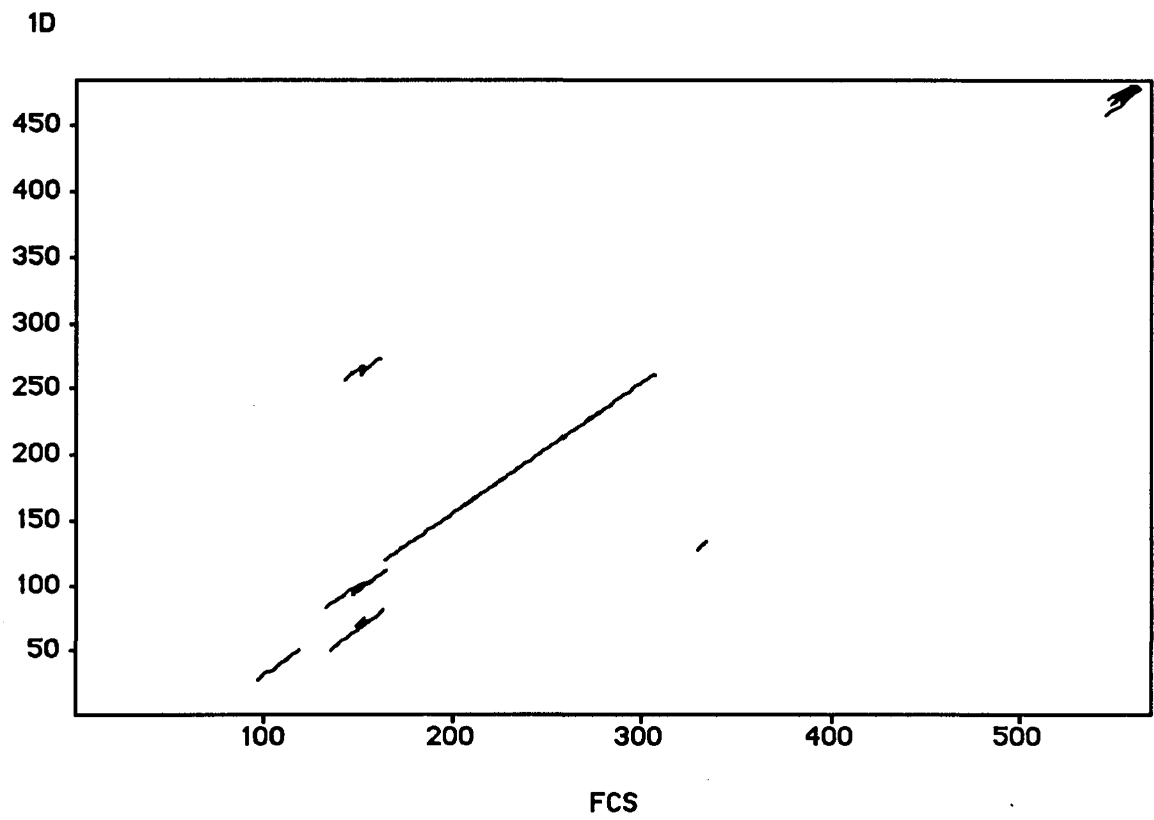

FIG. 12. Matrix analysis of the FCS and 1D genes.

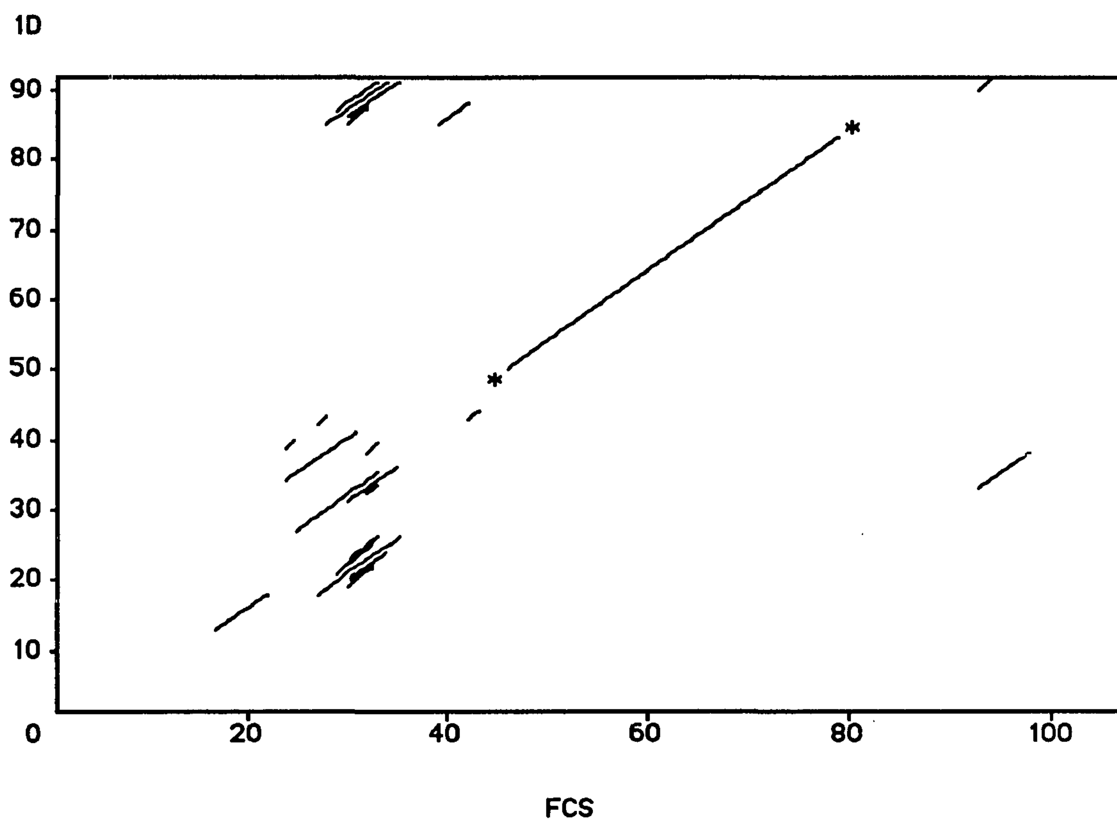

FIG. 13. Matrix analysis of the deduced peptide sequences of FCS and 1D. The highest scoring local alignment is indicated with asterisks.

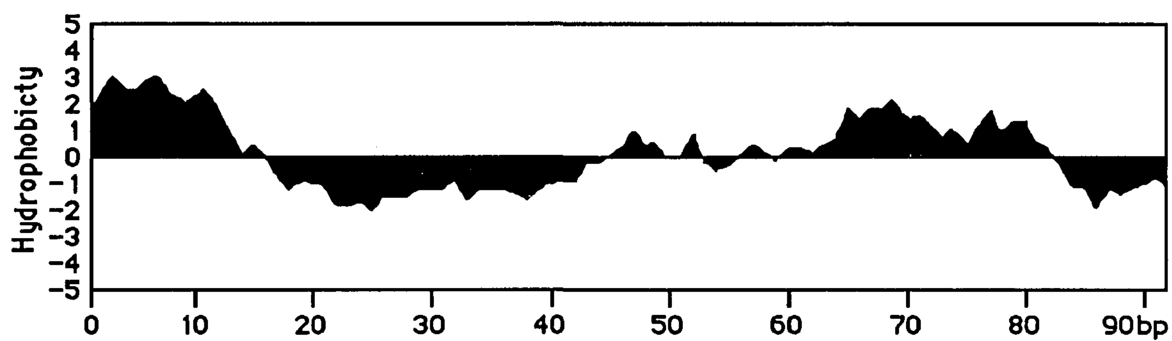

FIG. 14. Hydropathy plot analysis of the deduced 1D peptide sequence according to the method of Kyte and Doolittle (1982).

TABLE 1

COMPARISON OF AMINO ACID CONTENTS OF FCS TRANSLATION PRODUCT AND *DROSOPHILA* VITELLINE MEMBRANE PROTEINS (RESIDUES/100 RESIDUES)

Residue	FCS*	26A*	32E*	34C*	TU4*
Ala	16.7	10.2	17.2	21.1	28.3
Cys	3.3	2.5	4.0	4.8	1.4
Asp	3.3	1.7	0.0	0.0	0.0
Glu	3.3	1.7	5.1	0.0	1.4
Phe	2.2	1.7	2.0	2.9	1.4
Gly	5.6	14.4	8.1	6.7	4.1
His	14.4	0.9	1.0	0.1	0.0
Ile	0.0	0.9	2.0	4.8	3.4
Lys	2.2	1.7	0.0	4.8	0.7
Leu	4.4	5.1	6.1	5.8	4.8
Met	0.0	0.0	2.0	2.9	0.0
Asn	1.1	2.5	3.0	0.0	1.4
Pro	23.3	11.0	14.1	16.3	19.3
Gln	1.1	7.6	11.1	0.0	6.2
Arg	1.1	1.7	3.0	2.9	2.1
Ser	3.3	13.6	7.1	10.6	11.7
Thr	1.1	6.8	5.1	1.0	0.7
Val	7.8	5.9	1.0	3.8	3.4
Trp	0.0	0.0	1.0	0.0	0.0
Tyr	5.6	10.2	7.1	4.8	9.7

*Deduced composition from DNA sequence.

TABLE 2

COMPARISON OF AMINO ACID CONTENTS OF FCS TRANSLATION PRODUCT AND OTHER INSECT EGGSHELL PROTEINS (RESIDUES/100 RESIDUES)

Residue	FCS[a]	VMP[b]	Chorion[c]	Eggshell[d]
Ala	16.7	28.6	16.8	12.1
Cys	3.3	1.0	2.7	6.4
Asp	3.3	0.0	5.1	-
Glu	3.3	0.0	4.0	0.0
Phe	2.2	1.8	1.9	1.4
Gly	5.6	10.4	21.8	32.6
His	14.4	0.4	0.0	0.0
Ile	0.0	1.3	3.3	3.8
Lys	2.2	3.0	0.5	0.5
Leu	4.4	3.4	7.2	7.6
Met	0.0	0.0	1.0	0.4
Asn	1.1	0.0	-	-
Pro	23.3	18.3	6.7	4.4
Gln	1.1	0.0	-	-
Arg	1.1	0.0	3.1	2.3
Ser	3.3	16.9	6.2	3.7
Thr	1.1	0.9	4.5	3.0
Val	7.8	3.0	8.3	6.5
Trp	0.0	0.0	1.1	1.0
Tyr	5.6	3.5	5.6	6.4
Glu+Gln	4.4	4.4	-	-
Asp+Asn	4.4	4.2	-	-

[a]Deduced protein from DNA sequence (this paper).
[b]Vitelline membrane protein of *Drosophila melanogaster* (Petri *et al.*, 1976).
[c]Class C chorion protein (Regier *et al.*, 1983).
[d]Eggshell protein of *Antheraea polyphemus* (Kafatos *et al.*, 1977).

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