INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600
VITELLINE MEMBRANE GENES IN THE YELLOW FEVER MOSQUITO,

*Aedes aegypti*

by

Marten John Edwards

Copyright © Marten John Edwards 1996

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF ENTOMOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1 9 9 6
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Marten John Edwards entitled Vitelline Membrane Genes in the Yellow Fever Mosquito, Aedes aegypti and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Henry H. Hagedorn  
Date 2/25/96
Reginald F. Chapman  
Date 2/4/96
David N. Byrne  
Date 2/9/96
Jenifer B. Feyereisen  
Date 2/9/96
John H. Law  
Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director  
Date 2/25/96
Henry H. Hagedorn
STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under the rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the copyright holder.

SIGNED: [Signature] 4/25/96
ACKNOWLEDGMENTS

I sincerely thank my advisor and mentor Henry H. Hagedorn for the insights, assistance, persistence, and encouragement throughout the entire process of researching and writing this dissertation.

I thank the members of my dissertation committee, Reginald F. Chapman, Michael A. Wells, John H. Law, Rene Feyerisen and David N. Byrne for their valuable suggestions, guidance and for helping to provide an intellectually stimulating and challenging academic environment. Thanks to the members of the Hagedorn lab, Craig Cady, Zhijian Tu, Jun Isoe and Julia Guzova, for the good times we shared. Thanks to Skip Vaught at the Macromolecular Structures Facility for the DNA sequencing. Many thanks to the first class administrative staff of the Department of Entomology and the Center for Insect Science, especially Sharon Richards, Justine Collins, Rose Kilby and Marylou Stengel. Special thanks to Amy Faivre.
TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................... 9

LIST OF TABLES ............................................................................................................. 11

ABSTRACT ..................................................................................................................... 12

I. THE VITELLINE MEMBRANE .................................................................................. 13
   ABSTRACT ................................................................................................................ 13
   Nomenclature of the vitelline membrane ................................................................. 13
   Formation and ultrastructure of the vitelline membrane ..................................... 15
   Aedes aegypti vitelline membrane genes .............................................................. 16
   Regulation of A. aegypti vitelline membrane gene expression ............................. 17
   Drosophila melanogaster vitelline membrane genes ............................................. 18
   Regulation of D. melanogaster vitelline membrane genes .................................... 20
   Relationships between the vitelline membrane genes ........................................ 22
   Conserved regions ................................................................................................ 23
   Amino acid composition ...................................................................................... 23
   Relationship to the chorion ................................................................................ 24
   Conclusions .......................................................................................................... 25

II. THE PHYSIOLOGY OF EGG DEVELOPMENT .................................................... 31
   SUMMARY .............................................................................................................. 31
   Stages of egg development ................................................................................. 32
   The role of hormones ......................................................................................... 34
   The ecdysone receptor ....................................................................................... 36
   The ecdysone responsive element ..................................................................... 36
   The physiology of chorion formation ............................................................... 37
   The Drosophila melanogaster chorion ............................................................... 38
   The silkmoth chorion ......................................................................................... 39
### TABLE OF CONTENTS - Continued

#### III. SEQUENCE ANALYSIS OF THREE VITELLINE MEMBRANE GENES IN THE YELLOW FEVER MOSQUITO, *Aedes aegypti*

- ABSTRACT ................................................................. 44
- INTRODUCTION ........................................................... 45
- METHODS ................................................................. 46
  - Chemicals and cloning vectors .................................. 46
  - Genomic library screening ....................................... 46
  - DNA sequencing ...................................................... 46
- RESULTS ................................................................. 48
  - Selection and identification of genomic clones ............ 48
  - Sequence of the genomic clones ............................... 49
  - Predicted promoter regions ..................................... 49
  - Analysis of the predicted 15a-3 transcriptional unit .. 50
  - Conserved regions .................................................. 50
  - Potential ecdysone responsive elements ..................... 51
- DISCUSSION ........................................................... 52
  - 15a-3 is a vitelline membrane gene ......................... 53
  - Conserved regions .................................................. 54
  - Proline content ..................................................... 54
  - Analysis of the upstream regions ............................. 54
  - Potential ecdysone response elements ....................... 56
IV. REGULATION OF VITELLINE MEMBRANE GENE EXPRESSION IN THE YELLOW FEVER MOSQUITO, *Aedes aegypti* .......................................................... 68

ABSTRACT ............................................................... 68
INTRODUCTION .......................................................... 69

METHODS .................................................................... 70
Animals ...................................................................... 70
Chemicals .................................................................... 70
Hybridization probes ..................................................... 70
RNA isolation and characterization ................................. 71
Whole-mount *in situ* hybridization ................................. 72
*In vitro* culture of ovaries .............................................. 73

RESULTS .................................................................... 74
Timing of expression ...................................................... 75
Sites of expression ......................................................... 75
Effect of *in vitro* culture .................................................. 75
Effect of ecdysone *in vitro* .............................................. 76

DISCUSSION .............................................................. 79
Timing of expression ...................................................... 79
Spatial expression .......................................................... 79
Role of ecdysone ............................................................. 80
Effect of ecdysone *in vitro* .............................................. 82

V. REPORTER CONSTRUCTS ........................................ 91

ABSTRACT ................................................................... 91
INTRODUCTION ........................................................... 92

METHODS .................................................................... 93
Vectors ....................................................................... 94
Preparation of the 15a-1 construct .................................. 94
Preparation of the 15a-2 construct .................................. 95
Preparation of the 15a-3 construct .................................. 96
TABLE OF CONTENTS - Continued

VI. MAPPING THREE VITELLINE MEMBRANE GENES
    ONTO THE Aedes aegypti CHROMOSOMES................................. 102
    INTRODUCTION......................................................................... 102
    METHODS............................................................................... 103
    RESULTS............................................................................... 104

VII. SUMMARY and SUGGESTIONS FOR FURTHER STUDY.............. 105
    SUMMARY............................................................................... 105
    SUGGESTIONS FOR FURTHER STUDY.................................. 108
        Mobility shift assays......................................................... 108
        In vitro methylation interference assays......................... 109
        DNase I footprinting......................................................... 109

VIII. REFERENCES........................................................................ 110
LIST OF FIGURES

FIGURE 1.1, Alignment of the protein sequences of the conserved regions in the *A. aegypti* and *D. melanogaster* vitelline membrane genes ......................................................29

FIGURE 1.2, Hydropathy analysis of the deduced *A. aegypti* and *D. melanogaster* vitelline membrane peptides according to the method of Kyte and Doolittle (1982) ..................30

FIGURE 2.1, Diagrammatic representation of hormone changes from 0 to 6 days after the emergence of the adult female .................................................................42

FIGURE 2.2, Model for the control of egg development in *A. aegypti* ........................................43

FIGURE 3.1, Restriction maps and sequencing strategy of the 15a-1, 15a-2 and 15a-3 genomic clones .................................................................58

FIGURE 3.2, Nucleotide sequences within three *A. aegypti* vitelline membrane clones a) 15a-1 b) 15a-2 and c) 15a-3 ......................................................62

FIGURE 3.3, Promoter regions of 15a-1, 2 and 3 showing conserved TATA box and arthropod initiator sequences in bold ........................................... 63

FIGURE 3.4, Hydropathy analysis of 15a-3 open reading frame ......................................................64

FIGURE 3.5, Multiple sequence alignment of the predicted mature peptide sequences from the 15a-1, 15a-2 and 15a-3 genes ...........................................65

FIGURE 3.6, Alignment of the protein sequences of the conserved regions in the *A. aegypti* and *D. melanogaster* vitelline membrane genes ...........................................66

FIGURE 3.7, Similar region contained in both 15a-2 and *A. aegypti* vitellogenin gene VgA1 upstream regions ...........................................67
LIST OF FIGURES, CONTINUED

FIGURE 4.1, Mutually exclusive probes for 15a-1, 15a-2 and 15a-3 85

FIGURE 4.2, Northern blot showing timing of vitelline membrane gene expression in ovaries following a blood meal 86

FIGURE 4.3, Graph of vitelline membrane gene expression in ovaries following a blood meal 87

FIGURE 4.4, Sites of 15a-1, 15a-2 and 15a-3 expression in ovaries at 24 and 36 h PBM 89

FIGURE 4.5, Effect of in vitro culture and ecdysone on vitelline membrane gene expression at increasing intervals after a blood meal 90

FIGURE 5.1, Reporter construct containing DNA sequence upstream of the A. aegypti vitellogenin gene VgA1 97

FIGURE 5.2, Cloning strategy for reporter constructs 98

FIGURE 5.3, Reporter construct containing DNA sequence upstream of vitelline membrane gene 15a-1 99

FIGURE 5.4, Reporter construct containing DNA sequence upstream of vitelline membrane gene 15a-2 100

FIGURE 5.5, Reporter construct containing DNA sequence upstream of vitelline membrane gene 15a-3 101
LIST OF TABLES

TABLE 1.1, Percent similarities between *A. aegypti* and *D. melanogaster* vitelline membrane genes ........................................ 26

TABLE 1.2, Adjusted qualities of comparisons of *A. aegypti* and *D. melanogaster* vitelline membrane genes .............................. 27

TABLE 1.3, Comparison of the deduced content of selected amino acids of the *A. aegypti* and *D. melanogaster* vitelline membrane genes ........................................ 28

TABLE 3.1, Potential ecdysone responsive elements (EcREs) upstream of 15a-1, 15a-2 and 15a-3 ................................................................. 57

TABLE 4.1, CPM values for time course Northern blot ......................................................................................... 83

TABLE 4.2, Effect of *in vitro* culture and ecdysone on 15-1 expression at increasing intervals PBM ..................................................... 84
ABSTRACT

Three vitelline membrane (envelope) genes in Aedes aegypti mosquitoes were studied. A genomic clone corresponding to a novel vitelline membrane gene (15a-3) was isolated. The predicted peptide sequence of 15a-3 is similar to those of the A. aegypti vitelline membrane genes 15a-1 and 15a-2. The deduced amino acid sequences of 15a-1, 15a-2 and 15a-3 contain a conserved region of 45 residues. This region overlaps with a conserved region in four Drosophila melanogaster vitelline membrane genes. Genomic clones corresponding to 15a-1 and 15a-2 were isolated that contained the complete 15a-1 and 15a-2 transcriptional units. DNA regions of approximately 1.8 kb, 2.1 kb and 2.0 kb were sequenced upstream of the 15a-1, 15a-2 and 15a-3 coding regions. A 360 bp region 5' of the 15a-2 coding region was identified with 72% identity to a region upstream of the A. aegypti VgA1 vitellogenin gene. Sequences with similarity to a 20-hydroxyecdysone response element consensus sequence were found upstream of the three coding regions. The transcriptional regulation of three A. aegypti vitelline membrane genes was examined. The sites and timing of expression of the three transcripts were determined by Northern blot analysis and whole-mount in situ hybridization using mutually exclusive probes. The temporal pattern of 15a-1, 15a-2 and 15a-3 expression was similar. The spatial pattern of expression differed between the three genes. Only 15a-2 was expressed at the anterior region in addition to the remainder of the follicle. 15a-1 and 15a-3 were only expressed in the mid and posterior regions of the follicle. Expression of 15a-1 was higher in ovaries that were dissected at intervals of 0, 2, 10 and 24 h after a blood meal and were cultured in medium containing 20-hydroxyecdysone, as compared to control incubations. In ovaries that were dissected at 36 h after a blood meal, incubation in 20-hydroxyecdysone had no effect on 15a-1 expression, as compared to control incubations.
CHAPTER I

THE VITELLINE MEMBRANE

ABSTRACT

The insect eggshell provides a model system for the study of gene regulation because several proteins are synthesized in an ordered spatial and temporal pattern within a single tissue, the follicular epithelium. Progress is being made towards an understanding of *A. aegypti* and *D. melanogaster* vitelline membrane formation. The vitelline membrane is the innermost layer of the eggshells of *A. aegypti* and *D. melanogaster*. Genes encoding three *A. aegypti* and four *D. melanogaster* vitelline membrane proteins have been cloned and sequenced. Significant similarity is observed between the *A. aegypti* and *D. melanogaster* vitelline membrane genes. The regulation of vitelline membrane gene expression in *A. aegypti* and *D. melanogaster* is compared.

Nomenclature of the vitelline membrane

The vitelline membrane is the innermost layer of the *A. aegypti* and *D. melanogaster* eggshell. It a product of the follicular epithelium in both *A. aegypti* (Mathew and Rai, 1975; Raikhel and Lea, 1982, 1991) and *D. melanogaster* (Mahowald, 1972; Mahowald and Kambysellis, 1980; Fargnoli and Waring, 1982). In *A. aegypti*, this layer is sometimes called the endochorion (Powell et al. 1988; Clements, 1992) to avoid confusion with usage of the term vitelline membrane to identify an extracellular matrix that is secreted by the oocyte in many organisms. The vitelline membrane of echinoderms,
birds and amphibians for example, is a product of the oocyte (Smiley, 1990; Hopper and Hart, 1985).

The usage of the term “vitelline membrane” in the Diptera reflects an early misconception that the vitelline membrane was product of the oocyte, for example by Nath (1924). Usage of the term endochorion instead of vitelline membrane is confusing because the term endochorion is used to describe a different eggshell layer in *A. aegypti* (Mathew and Rai, 1975) and in *D. melanogaster* (Margaritis, 1985). Other authors use the term "vitelline envelope" to distinguish the innermost eggshell layer from a lipid bilayer or plasma membrane (Raikhel and Lea, 1982, 1991; Mazzini et al. 1987).

In spite of these drawbacks, vitelline membrane is the most frequently used term to describe the innermost eggshell layer of many orders of insects, including several lepidopteran (Zimowska et al. 1995; Mazur et al. 1989) and dipteran species (Margaritis, 1985). This term is employed in all of the literature describing the molecular biology of the structure, thus it is used in this dissertation. Unfortunately, the terminology of insect eggshell morphology does not address the question of whether the synonymous structures in different insect groups are composed of evolutionarily related proteins. It is possible that the innermost eggshell structures have arisen independently multiple times. The issue of homology will require a comparative study within a phylogenetic context. As eggshell genes from diverse insect orders are cloned and sequenced, a re-evaluation of the current system of nomenclature may be possible.
Formation and ultrastructure of the vitelline membrane

Unlike *D. melanogaster* follicles, which can be found at all stages of development in the adult fly, mosquito follicles develop synchronously. In *A. aegypti*, a blood meal initiates the development of about 75 to 150 eggs from a resting state to maturity within 3 days. For this reason, the formation of the *D. melanogaster* vitelline membrane will be discussed in terms of the oogenic stage of the follicle (Margaritis, 1985), whereas the formation of the *A. aegypti* vitelline membrane will be discussed in the context of the time elapsed since a blood meal.

The *A. aegypti* vitelline membrane first appears 8 to 10 h post-blood-meal (PBM) as discreet protein droplets called "vitelline bodies" or "plaques". The vitelline bodies are at first approximately 0.4 microns in diameter and are interdigitated between adjacent oocyte microvilli (Mathew and Rai, 1975; Raikhel and Lea, 1991). Between 18 and 30 h following a blood meal, the vitelline bodies increase in size from 0.4 to 1.2 microns in diameter (Raikhel and Lea, 1991).

By 40 hours PBM, the vitelline bodies have fused into a single homogeneous envelope approximately 1.2 μm thick (Mathew and Rai, 1975). The precipitous decline in yolk uptake by the oocyte around 32 to 36 hours PBM occurs before the vitelline bodies have coalesced. The passage of yolk components from the hemolymph into the egg is prevented by closing the channels between follicular cells by tight junctions [desmosomes] (Anderson and Spielman, 1971; Raikhel, 1992).

The formation and ultrastructure of the *D. melanogaster* vitelline membrane is similar to *A. aegypti*. In *D. melanogaster*, vitelline bodies are secreted around the oocyte by stage 9A (Margaritis, 1985). The vitelline bodies fuse at stage 10B and make a 1.7 micron thick vitelline membrane, which gradually thins down to a uniform layer about 0.3 microns thick as oogenesis proceeds (Margaritis, 1985). Unlike *A. aegypti*, the formation
of the *D. melanogaster* vitelline membrane is followed by the deposition of an impermeable wax layer between it and the chorion. The wax layer presumably protects the embryo from desiccation. The wax layer of *D. melanogaster* eggs is of practical significance, as it allows for the dechorionation of eggs in genetic transformation experiments. The remarkable resistance to desiccation observed in *A. aegypti* eggs is provided by the serosal cuticle which is secreted by the embryo and becomes impregnated with wax (Beckel, 1958; Clements, 1992). This structure is often curiously referred to as the vitelline membrane in the mosquito literature (Telford, 1957; Moretti and Larson, 1973; Raminani and Cupp, 1978).

**Aedes aegypti vitelline membrane genes**

An *A. aegypti* vitelline membrane gene (15a-1) was cloned by a differential screen of an *A. aegypti* genomic library by Gemmill et al. (1986). Using a radiolabeled restriction fragment of the 15a-1 genomic clone, Lin et al. (1993) isolated a corresponding clone from a cDNA library prepared from blood-fed female mosquitoes. Sequence analysis of the 15a-1 cDNA clone indicated that it contained an open reading frame encoding a predicted mature peptide of 9.5 kDa.

A partial cDNA clone (15a-2) that hybridized with a 15a-1 coding region probe was also isolated by Lin et al. (1993). The 15a-2 sequence contained a partial open reading frame encoding a predicted mature peptide of 8.1 kDa. A genomic clone (15a-3) that hybridized with a 15a-2 coding region probe was isolated by Edwards and Hagedorn (unpublished observations). The predicted translation product of 15a-3 has an open reading frame encoding a predicted mature peptide of 11.1 kDa. The percent amino acid similarities between 15a-1, 2 and 3 are shown in Table 1.1.
Powell et al (1988) have described peptides isolated from the chorion of *A. aegypti*, which presumably included components of the vitelline membrane. Two groups of peptides (A1/A2 and C1-C4 peptides) appear between 24 and 48 hr after the blood meal. Because of the timing of their appearance, these are the most likely candidates for being part of the vitelline membrane. Powell et al. (1987) did not observe any peptides in the size range of the 15α-l, 15α-2 or 15α-3 vitelline membrane gene open reading frames. The smallest peptides they found were the C1-C4 class which were about 18 kDa which may be dimers of a 15α-l, 2 or 3 gene product. The approximately 90 kDa A1/A2 peptides could also be multimers of these gene products.

**Regulation of *Aedes aegypti* vitelline membrane gene expression**

There is evidence that formation of the *A. aegypti* vitelline membrane is regulated by 20-hydroxyecdysone (ecdysone). Raikhel and Lea (1991) have shown that the secretion of a morphologically normal vitelline membrane (envelope) can be induced in female mosquitoes with two 500 pg injections of ecdysone, spaced 12 hours apart. These mosquitoes were also given blood by enema, and decapitated. Injections with ecdysone were only effective in mosquitoes with blood in the midgut. Decapitation prevents the release of the egg development neurosecretory hormone which stimulates production of ecdysone by the ovaries (Hagedorn et al. 1979). No vitelline membrane plaques were observed in these mosquitoes when they were injected with saline (Raikhel and Lea, 1991).

Lin et al. (1993) demonstrated by Northern analysis that 15α-l message was expressed in the ovaries of blood-fed, decapitated females 24 hours following the injection of 1 μg of ecdysone in saline. No expression was observed in saline injected controls. Expression of the 15α-l gene was also detected in ovaries dissected from non-blood fed female mosquitoes (3 days post-emergence) that were cultured for 24 hours in medium
containing $10^{-5}$ M ecdysone. No message was detected in ovaries that were cultured in medium without ecdysone (Lin et al. 1993).

In terms of the physiology of *A. aegypti*, it is reasonable that ecdysone has a role in vitelline membrane formation. The appearance of this structure coincides with a rise in ecdysone titer that occurs from 3 to 48 hours and peaks between 16 and 20 hrs PBM (Hagedorn et al. 1975; Racioppi et al. 1986). This hormone is involved in the coordinated development of eggs from a resting stage to maturity within three days. Several studies have demonstrated that ecdysone stimulates the expression of vitellogenin genes in the fat body of *A. aegypti* (Racioppi et al. 1986; Ma et al. 1987; Dietsch et al. 1995).

**Drosophila melanogaster** vitelline membrane genes

Petri et al. (1976) suggested that four size classes of vitelline membrane proteins include ten or more distinct proteins. At least six major size classes of vitelline membrane proteins were observed by Fargnoli and Waring (1982) and Mindrinos et al. (1985). Genes encoding four vitelline membrane proteins have been cloned and sequenced. Since the nomenclature of the *D. melanogaster* vitelline membrane clones is inconsistent in the literature, the clone designations of Jin and Petri (1994) will be used. Accordingly, the sequence of the VM26A1 gene was reported by Burke et al. (1987), VM26A2 by Popodi et al. (1988), VM32E by Gigliotti et al. (1989). A partial cDNA clone of VM34C was reported by Mindrinos et al. (1985), which was subsequently completed with a genomic sequence by Scherer et al. (1993).

The VM26A1 clone was obtained by screening an ovarian cDNA library with vitellogenic egg chamber RNAs labeled *in vivo* (Burke et al. 1987). Hybrid selection experiments of Popodi et al. (1988) demonstrated that the translation product of this gene
comigrated with the 17.5 kDa vitelline membrane protein (Sv17.5) described by Fargnoli and Waring (1982). Expression of the VM26A1 message was demonstrated by in situ hybridization in the follicular epithelium during the period of vitelline membrane formation. Furthermore, the amino acid composition of this peptide was consistent with the amino acid composition of vitelline membrane proteins as measured by Petri et al. (1976). A corresponding VM26A1 genomic clone was localized by in situ hybridization to chromosomal location 26A (Burke et al. 1987). This region was previously shown to contain sequences that were complementary to mRNA messages expressed in vitellogenic egg chambers during the time of vitelline membrane synthesis (Higgins et al. 1984; Mindrinos et al. 1985).

Popodi et al. (1988) hybridized subcloned restriction fragments of chromosomal region 26A with stage 10 egg chamber mRNA. This led to the cloning of VM26A2 (TU4). Hybrid selection experiments showed that the translation product of this gene comigrates with a vitelline membrane protein (Sv23), described by Fargnoli and Waring (1982). A female sterile mutant that was defective in vitelline membrane formation was identified by Savant and Waring, (1989). This mutant failed to produce the Sv23 protein and the site of this mutation was mapped cytogenetically to chromosomal region 26A. The site of VM26A2 expression was demonstrated by in situ hybridization in the follicular epithelium during the time of vitelline membrane formation. Furthermore, its amino acid composition was consistent with vitelline membrane proteins described by Petri et al. (1976). VM26A2 was localized in the chromosome within four kilobases and in the opposite orientation of VM26A1 (Popodi et al. 1988).

The 26A region of the second chromosome is not the only place where vitelline membrane genes can found, as demonstrated by Mindrinos et al. (1985). An all-stage egg chamber cDNA library was differentially screened with cDNA derived from stage 1-10
and stage 11-14 follicles. Two clones were isolated, and subsequent northern analysis revealed that they were only expressed during stages 8, 9 and 10. One of these clones, VM34C (DmcMM99), was sequenced and the sequence was consistent with the expected amino acid composition of a vitelline membrane gene (Petri et al. 1976). This clone was localized to polytene band location 34C. A corresponding genomic clone was isolated and sequenced by Scherer et al. (1993).

Mindrinos et al. (1985) noted that VM34C also hybridized to chromosomal site 32EF under low stringency conditions. A vitelline membrane gene (VM32E) was isolated from this region by Gigliotti et al. (1989). A subcloned fragment of region 32E that hybridized with an adult female cDNA clone contained a region of significant similarity to the other vitelline membrane genes (Tables 1.1 and 1.2).

The site and timing of VM32E expression is consistent with that expected of a vitelline membrane gene, although not identical to the previously isolated vitelline membrane genes (Gigliotti et al. 1989; Gargiulo et al. 1991). VM32E is only expressed in the main columnar follicular cells and not in the anterior or posterior cells. Interestingly, VM32E expression was first detected at stage 10A egg chambers in a small group of ventral columnar cells (Gargiulo et al. 1991). This timing of expression is significantly different from other vitelline membrane genes, eg. VM26A1 and VM32C, where expression can be detected as early as stage 8 (Mindrinos et al. 1985; Burke et al. 1987).

**Regulation of the *D. melanogaster* vitelline membrane genes**

The regulation of the *D. melanogaster* vitelline membrane genes has been studied by the analysis of DNA sequences upstream of their respective coding regions. Savant and Waring (1989) demonstrated that transposons carrying the VM26A2 (Sv23) gene with as little as 147 bp of 5' flanking DNA were capable of restoring fertility to a vitelline
membrane mutant of *D. melanogaster*. In transformed flies, expression of the Sv23 protein was restored to wild type levels. Thus, all of the necessary *cis*-acting regulatory elements for wild type expression were located immediately upstream of the VM26A2 coding region.

Jin and Petri (1993) analyzed the regulation of VM26A1. DNA fragments from the 5' flanking region of this gene were fused to hsp/lacZ genes, reintroduced via P-element transformation, and functionally assayed by histological staining for β-galactosidase activity. They demonstrated that 597 bp of 5' flanking DNA is sufficient to promote the expression of a lacZ reporter gene with sex, tissue and stage specificities identical to those characteristic of endogenous VM26A1 gene expression. A developmental control element capable of promoting reporter gene expression specifically in the follicular epithelium of stage 8, 9 and 10 egg chambers was found within a 176 bp region of this sequence. Three additional control elements were identified that appropriately modulated the spatial expression of constructs containing the core element in their promoter region.

Gargiulo et al. (1991) characterized two *cis*-acting regulatory sequences in the upstream region of VM32E in P-element transformation experiments. They showed that the first 465 nucleotides upstream of VM32E confer a high level of appropriate stage and tissue specific expression to a reporter gene, β-galactosidase. A proximal element within this region confers temporal specificity and a distal element amplifies the level of expression. Gargiulo et al. (1991) reported sequence similarities to an ecdysone response element upstream of VM32E, however the sequence does not resemble the consensus sequence for the ecdysone responsive element (Antoniewski et al. 1994). In contrast to *A. aegypti*, no role has been shown for ecdysone in the regulation of *D. melanogaster* vitelline membrane genes.
The comparative analysis of three *D. melanogaster* vitelline membrane upstream sequences (VM26A1, VM34C and VM32E) revealed a heptameric sequence (AAGTGC) (Scherer et al. 1993). This consensus sequence is also present upstream of the *D. melanogaster* yolk protein, YP1-3. All of these regions confer ovarian temporal and tissue-specific expression to their respective transcriptional units. The significance of this heptameric sequence has not yet been determined by any functional assay.

**Relationships between the vitelline membrane genes**

The deduced peptide sequences of the *A. aegypti* vitelline membrane genes were used to search the NBRF protein data base using the FastA program (Pearson and Lipman, 1988). Similarity was detected between the *A. aegypti* and the *D. melanogaster* vitelline membrane genes. The percent similarities between the protein sequences were determined by the GCG Gap program allowing for a gap penalty of 3.0 (Table 1.1).

The statistical significance of the comparisons between the *A. aegypti* and *D. melanogaster* vitelline membrane genes was determined calculating an adjusted quality (Q') of the comparison by the formula of Barker and Dayhoff (1972) which is shown below. The adjusted quality is an index of the probability that the quality of the tested comparison could be obtained by random chance. The probability of obtaining the adjusted score can be determined from a z-score table. For example, the probability of obtaining a score of 3.0 is 0.13%. An adjusted score of 3.0 or greater is required for a reasonable confidence that two sequences are significantly related (Barker and Dayhoff, 1972; Romans et al. 1995). Quality scores (Q) were calculated by the GCG Gap program, allowing for a gap penalty of 3.0.

\[
Q' = \frac{Q - \text{Mean } Q \text{ of 100 random comparisons}}{\text{Standard deviation of 100 random comparisons}}
\]
The adjusted qualities of all of the *A. aegypti* vs. *A. aegypti* and *D. melanogaster* vs. *D. melanogaster* comparisons are greater than three, thus they are significantly similar (Table 1.2). This is not the case with all of the *A. aegypti* vs. *D. melanogaster* comparisons (Table 1.2). For example, the *A. aegypti* 15a-3 gene is not similar to any of the *D. melanogaster* sequences ($Q' < 3.0$ for all comparisons). However, both *A. aegypti* 15a-1 and 15a-2 genes are significantly similar to two *D. melanogaster* vitelline membrane genes. It appears that the vitelline membrane genes of *D. melanogaster* and *A. aegypti* belong to superfamily of overlapping but distinct gene families (Doolittle, 1981). The sequence similarity between these genes suggests that the vitelline membranes of these insects are evolutionary conserved.

**Conserved regions**

A common feature of the four *D. melanogaster* vitelline membrane gene sequences is a similar 114 base pair region that is located in the coding regions of the genes (Scherer et al. 1988) (Fig. 1.1). In this region, the predicted peptide sequences of VM26A1 and VM34C are identical, whereas VM32E is relatively divergent from the other three sequences. A similar region is present in the *A. aegypti* genes 15a-1 and 15a-2 (Lin et al. 1993) (Fig. 1.1). This region is also contained with significant similarity in the *A. aegypti* 15a-3 sequence (Edwards and Hagedorn, unpublished observations).

An interesting difference between the *A. aegypti* and the *D. melanogaster* conserved regions is their position in relation to the full-length protein sequence. Whereas the relative positions of the *A. aegypti* conserved regions are similar, this is not the case with the *D. melanogaster* vitelline membrane genes. In particular, the conserved region of VM32E is located near the 5' terminus of the protein. The relative positions of the conserved regions are highlighted in the hydrophilicity plots of these sequences (Fig. 1.2).
The hydrophilicity plots also show that whereas the overall pattern of hydrophilicity is conserved between all three *A. aegypti* vitelline membrane genes, this is not the case with the *D. melanogaster* vitelline membrane genes which differ greatly in their hydrophilicity plots downstream of the signal sequences (Fig. 1.2).

**Amino acid composition**

The deduced amino acid compositions of selected residues in the *A. aegypti* and *D. melanogaster* vitelline membrane genes are compared in Table 1.3. Both the *A. aegypti* and *D. melanogaster* sequences are rich in both alanine and proline when compared to the average composition of these residues in all proteins (Dayhoff, 1978). However, the *A. aegypti* sequences are relatively high in histidine and low in serine. The reciprocal is true of the *D. melanogaster* sequences which are relatively enriched in serine but have fewer histidine residues. The deduced amino acid composition of the *D. melanogaster* vitelline membrane genes is remarkably consistent with the empirical data from protein analysis of Petri et al. (1976) which are also shown in Table 1.1.

**Relationship to the chorion**

In *D. melanogaster*, six major chorion proteins have been cloned and sequenced (Wong et al. 1985; Levine and Spradling, 1985; Spradling et al. 1987; Fenerjian et al. 1989). There is no significant similarity between the *D. melanogaster* vitelline membrane and chorion gene sequences. In the light of the sequence data, the cross-hybridization observed between VM26A1 and VM26A2 probes and s18-1 chorion gene mRNA (Higgins et al. 1984; Popodi et al. 1988) does not suggest that the two gene families are evolutionarily related.

The physical properties of the *D. melanogaster* vitelline membrane are different from the rest of the chorion. The empirically determined amino acid composition is
different between the two layers (Petri et al. 1976) (Table 1.3). Petri et al. (1979) demonstrated that the crosslinking of vitelline membrane and chorion proteins occurs independently during development. For example, cysteine bridges appear to be formed in the vitelline membrane at a time when the chorion is still soluble in 2% SDS.

Conclusions

Vitelline membrane genes have been cloned in *A. aegypti* and *D. melanogaster*. Significant similarity is observed between the two vitelline membrane gene families. A highly conserved region is contained within the *A. aegypti* and *D. melanogaster* vitelline membrane proteins. It is possible that sequences that are conserved between *A. aegypti* and *D. melanogaster* vitelline membrane genes may be present in other groups of insects. This would facilitate the isolation of novel vitelline membrane genes using heterologous DNA probes or by the polymerase chain reaction. This would address the question of whether the innermost eggshell layers of different groups of insects are homologous.

The regulation of *D. melanogaster* vitelline membrane genes has been studied in the context of gaining a comprehensive understanding of eggshell development. DNA sequences have been identified upstream of *D. melanogaster* vitelline membrane genes which confer tissue- and stage-specific expression. The regulation of *A. aegypti* vitelline membrane genes has also been investigated. Ecdysone has been shown to have a role in the expression of *A. aegypti* vitelline membrane genes.

Acknowledgments

This work was supported by NIH grant (HD24869) to Drs. Henry H. Hagedorn and Ann M. Fallon, and by the John D. and Catherine T. MacArthur Foundation.
TABLE 1.1. Percent similarities between *A. aegypti* and *D. melanogaster* vitelline membrane genes. References for the sequences are stated in the text. Similarities were determined using the GCG software package Gap program. A gap penalty of 3.0 was employed for all comparisons.

<table>
<thead>
<tr>
<th></th>
<th>15a-2</th>
<th>15a-3</th>
<th>VM26A1</th>
<th>VM26A2</th>
<th>VM32E</th>
<th>VM34C</th>
</tr>
</thead>
<tbody>
<tr>
<td>15a-1</td>
<td>84.3</td>
<td>91.7</td>
<td>44.8</td>
<td>54.3</td>
<td>53.0</td>
<td>57.1</td>
</tr>
<tr>
<td>15a-2</td>
<td>85.3</td>
<td>30.7</td>
<td>56.4</td>
<td>42.7</td>
<td>57.3</td>
<td></td>
</tr>
<tr>
<td>15a-3</td>
<td>33.0</td>
<td>50.0</td>
<td>40.4</td>
<td>47.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VM26A1</td>
<td></td>
<td></td>
<td>57.0</td>
<td>67.4</td>
<td>58.9</td>
<td></td>
</tr>
<tr>
<td>VM26A2</td>
<td></td>
<td></td>
<td>59.1</td>
<td>74.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VM32E</td>
<td></td>
<td></td>
<td></td>
<td>68.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 1.2. Adjusted qualities of comparisons of *A. aegypti* and *D. melanogaster* vitelline membrane genes. References for the sequences are stated in the text. The adjusted quality was determined using data calculated with the GCG sequence analysis software package and the Gap Program. An adjusted score of 3.0 or greater is required for a reasonable confidence that two sequences are significantly related. This value was calculated using the formula of (Barker and Dayhoff, 1977).

<table>
<thead>
<tr>
<th></th>
<th>15a-2</th>
<th>15a-3</th>
<th>VM26A1</th>
<th>VM26A2</th>
<th>VM32E</th>
<th>VM34C</th>
</tr>
</thead>
<tbody>
<tr>
<td>15a-1</td>
<td>12.2</td>
<td>22.3</td>
<td>1.8</td>
<td>2.3</td>
<td>3.8</td>
<td>4.8</td>
</tr>
<tr>
<td>15a-2</td>
<td>20.3</td>
<td>1.6</td>
<td>3.9</td>
<td>0</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>15a-3</td>
<td>1.5</td>
<td>2.7</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VM26A1</td>
<td>9.0</td>
<td>10.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VM26A2</td>
<td>5.5</td>
<td>14.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VM32E</td>
<td></td>
<td></td>
<td>11.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 1.3. Comparison of the deduced content of selected amino acids of the *A. aegypti* and *D. melanogaster* vitelline membrane genes. Also shown is the average amino acid composition of 314 representative protein sequences (Dayhoff et al. 1979) and the amino acid composition data of Petri et al. (1976). Figures are presented as the number of residues per 100 residues and have been rounded to the nearest whole number. (References: (Lin et al. 1993); 2. (Burke et. al. 1987); 3. (Popodi et al. 1988); 4. (Gigliotti et. al. 1989); 5. (Mindrinos et al. 1985); 6. (Petri et al. 1976); 7. (Dayhoff et al. 1979).)

<table>
<thead>
<tr>
<th>Species</th>
<th>Ref.</th>
<th>Protein(s)</th>
<th>Ala</th>
<th>Pro</th>
<th>His</th>
<th>Ser</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. aegypti</em></td>
<td>1</td>
<td>15a-1</td>
<td>17</td>
<td>23</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>15a-2</td>
<td>15</td>
<td>36</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>15a-3</td>
<td>13</td>
<td>31</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td><em>D. melanogaster</em></td>
<td>2</td>
<td>VM26A1</td>
<td>10</td>
<td>11</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>VM26A2</td>
<td>28</td>
<td>19</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>VM32E</td>
<td>17</td>
<td>14</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>VM34C</td>
<td>21</td>
<td>16</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td><em>D. melanogaster</em></td>
<td>6</td>
<td>Vitelline Membrane</td>
<td>29</td>
<td>18</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td><em>D. melanogaster</em></td>
<td>6</td>
<td>Chorion</td>
<td>15</td>
<td>11</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Several</td>
<td>7</td>
<td>Average of 314</td>
<td>9</td>
<td>5</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sequences</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 1.1. Alignment of the protein sequences of the conserved regions in the *A. aegypti* and *D. melanogaster* vitelline membrane genes. Conserved residues are shown in upper case letters. Residues that are conserved between the *A. aegypti* and *D. melanogaster* are indicated with a bar. Residues are numbered in reference to the translational start site as +1.
FIGURE 1. 2. Hydropathy analysis of the deduced *A. aegypti* and *D. melanogaster* vitelline membrane peptides according to the method of Kyte and Doolittle (1982). Plots were made using the MacVector (IBI) sequence analysis software. A window size of 7 was used. Conserved regions are highlighted in boxes.
CHAPTER II

THE PHYSIOLOGY OF EGG DEVELOPMENT

SUMMARY

The formation of the *Aedes aegypti* vitelline membrane is considered in the context of mosquito reproductive physiology. The vitelline membrane develops during just one of several oogenic stages that are reviewed with an emphasis on the morphological events that characterize them. Egg development in *A. aegypti* is regulated by the action of several hormones, including juvenile hormone and 20-hydroxyecdysone (ecdysone). The levels of these hormones are modulated by several peptide hormones, such as an egg development neurosecretory hormone (EDNH) and a releasing factor for EDNH (EDNH-RF). The formation of the vitelline membrane occurs during a peak of ecdysone. Ecdysone acts via a receptor molecule at ecdysone responsive elements. The formation of the *A. aegypti* chorion is compared to choriogenesis in *Drosophila melanogaster* and *Bombyx mori*. 
Stages of egg development

Egg development in *Aedes aegypti* proceeds through distinct developmental stages which are punctuated by a blood meal. The blood meal separates the previtellogenic from the vitellogenic (trophic) stages of egg development. The previtellogenic stage of egg development begins at eclosion of the adult female, however the process of egg development begins in 4th instar larvae with the development of a distinct pair of ovaries. All of the ovarian tissues are fully differentiated prior to the previtellogenic stage of egg development.

By the pupal stage, the paired, polytrophic ovaries contain about 70 ovarioles. Each ovariole consists of a mass of primordial germ cell nuclei surrounded by mesodermally derived nuclei that will differentiate into cells of the follicular epithelium. The germ line nuclei give rise to a cystocyte of eight cells through three incomplete mitotic divisions. One of the cells of the cystocyte will become an oocyte and the remaining seven will become nurse cells. During the pupal stage of development, the cystocyte becomes surrounded by follicular epithelial cells and forms a follicle. Within three days of adult emergence, one follicle, the primary follicle, from each of the ovarioles separates from the remaining follicles (reviewed in Clements, 1992).

During the first three days after eclosion, the primary follicles approximately double in length from about 50 \( \mu m \) to about 100 \( \mu m \) (Gwadz and Spielman, 1973). The cells of the follicular epithelium differentiate, their content of mitochondria, rough endoplasmic reticulum and Golgi complexes increases significantly (Raikhel and Lea, 1991). Three days after adult eclosion, the ovaries enter a "resting stage". No more changes will be observed in the ovaries for up to 10 days if the mosquito does not take a blood meal.
Immediately following the blood meal, several changes occur in the ovaries as they enter the initiation stage of egg development. Within an hour of the blood meal, channels (20 nm wide) appear between the cells of the follicular epithelium. These channels allow the passage of yolk materials from the hemolymph to the surface of the oocyte. Shortly after the blood meal, the oocyte surface becomes capable of taking up vitellogenin by endocytosis, however very little yolk material is endocytosed into the oocyte. Within two hours following the taking of a blood meal, a perioocytic space forms between the oocyte plasma membrane and the follicular epithelium (Raikhel and Lea, 1991).

The trophic phase of egg development occurs between approximately 8 and 48 h post blood meal (PBM). During this phase, vitellogenin synthesis occurs in the fat body. Synthesis starts at 3-4 h PBM, reaches its peak at 24-28 h PBM and then declines to background levels by 36-40 h PBM (Hagedorn et al., 1973; reviewed by Raikhel, 1992 and Hagedorn, 1995). Simultaneously, the oocyte length increases from approximately 80 μm to approximately 400 μm (Yonge and Hagedorn, 1977, Raikhel and Lea, 1991). The vitelline membrane is also formed during the trophic phase of egg development. At this time the structure is in the form of discreet plaques, which allow for the passage of yolk components into the oocyte.

It has been reported that the post-trophic phase of egg development starts when "plaques of endochorionic (vitelline membrane) material, secreted by the epithelial cells, fuse and block the endocytotic activities of the oocyte" (Clements, 1992). However, it has been shown that the precipitous decline in yolk uptake by the oocyte occurs before the vitelline bodies have coalesced. The passage of yolk components from the hemolymph into the egg is prevented by closing the channels between follicular cells by tight junctions (desmosomes) (Anderson and Spielman, 1971; Raikhel, 1992).
Several changes occur within the follicles during the post-trophic phase of egg development. The endochorionic and exochorionic layers of the *A. aegypti* eggshell are deposited by the follicular epithelium during this stage of development. The oocyte approximately doubles in size and assumes its final form. This increase in size is due to water and lipid uptake (Anderson and Spielman, 1971). The uptake of water and lipids presumably takes place in the anterior region of the oocyte, since the rest of the oocyte is surrounded by the chorion (Clements, 1992).

**The role of hormones**

The process of egg development in the adult is coordinated by several hormones, principally juvenile hormone III (JH) and 20-hydroxyecdysone (ecdysone). The levels of these hormones are regulated by the action of several peptide hormones. The relative levels of juvenile hormone and ecdysone during the adult life of the female mosquito are shown in Fig. 2.1. A model of how these hormones are involved in egg development is shown in Fig. 2.2.

Immediately after adult eclosion, JH is released from the corpora allata for two days. After this time, the level of JH slowly declines until a blood meal is taken. After a blood meal, the JH titre declines precipitously and stays low for 36 hours. At 36 h PBM, the level of juvenile hormone begins to rise again. It reaches its pre-blood meal titre at 66 h PBM (Shapiro et al., 1986). The second rise in JH titre is thought to be necessary to prepare the mosquito for a second blood meal (Gwadz and Spielman, 1973).

Juvenile hormone (JH) has many effects in the adult female during the previtellogenic stage of egg development. JH is necessary for the ovaries to become competent to respond to EDNH (Shapiro and Hagedorn, 1982). JH is also involved in the development of "competence" by the fat body to respond to ecdysone at later stages of
development (Flanagan and Hagedorn, 1988; Ma et al., 1988). The molecular basis of this competence to respond to ecdysone is not yet understood.

Juvenile hormone has pronounced effects on previtellogenic egg development. For example, when females are allatectomized at eclosion and their ovaries were examined 48 and 72 hours later, the ovaries remained small and undifferentiated in comparison to those of intact females of the same age. The effects of allatectomy were reversed by both the implantation of active corpora allata and the topical application of JH (Gwadz and Spielman, 1973; Raikhel and Lea, 1991).

The activity of JH degrading enzymes has shown to be important in the regulation of JH levels in the mosquito. Shapiro et al., (1986) found that JH esterase activity rose slowly for the first 12 h after a blood meal and then increased more rapidly to a peak at 42 h. The highest levels of JH esterase were seen when JH levels were the lowest. The blood meal also stimulates the release of an egg development neurosecretory hormone (EDNH) (also called the ovarian ecdisiotropic hormone) from the corpora cardiaca (Lea, 1967; Greenplate et al., 1985). The peptide(s), synthesized in the medial neurosecretory cells of the brain, stimulates the synthesis and secretion of ecdysone by the ovary (Hagedorn et al., 1979). Ecdysone from the ovary is converted to its active form, 20-hydroxyecdysone, by 20-monooxygenase in the fat body (reviewed by Hagedorn, 1985). Release of EDNH from the brain is mediated by a releasing factor (EDNH-RF) which is released from the ovaries following a blood meal (Lea and Van Handel, 1982). The titre of ecdysone released from the ovaries rises within 2-4 h PBM and reaches a peak around 24 h PBM. By 48 h PBM, the ecdysone titre returns its pre-blood meal level (Greenplate et al., 1985).
The ecdysone receptor

Ecdysone exerts its effects on transcriptional regulation via a receptor that is a member of a superfamily of steroid hormone receptors. The ecdysone receptor (EcR) binds DNA with high specificity at ecdysone responsive elements (EcREs). Koelle et al., (1991) cloned the *D. melanogaster* ecdysone receptor. The *D. melanogaster* EcR (dEcR) forms a heterodimer with the product of the gene, *Ultraspiracle*, which is homologous to the retinoid X receptor (Thomas et al., 1993).

An *A. aegypti* ecdysone receptor (AaEcR) corresponding to the *D. melanogaster* EcR (DmEcR) was cloned by Cho et al., 1995. The AaEcR clone has all of the characteristics that are predicted for a functional ecdysone receptor, with strong homology in each domain to the DmEcR. This clone was also highly homologous to a cDNA encoding a protein homologous to the ecdysone receptor, from *Chironomus tentans* by Imhof et al., (1993). In the fat body and ovaries of the female mosquito, the AaEcR mRNA peaks before a blood meal (1 and 2 days after eclosion) and 6 hr post blood meal. The concentration of the AaEcR mRNA was shown to be considerably higher in the ovaries than in the fat body, but the significance of this finding has yet to be established (Cho et al., 1995).

The ecdysone responsive element

The critical features of the EcRE of *D. melanogaster* were explored by Antoniewski et al., (1993). Gel mobility shift assays were performed using purified ecdysone receptors and oligonucleotides containing point mutations in a known ecdysone response element. Their results indicated that the sequence PuG(G/T)T(C/A)A(N)TG(C/A)(C/t)Py was the most conservative consensus sequence for the EcR-EcRE complex.
The presence of the EcRE consensus sequence within a putative regulatory region of an ecdysone responsive gene is not sufficient evidence that the sequence interacts with the ecdysone receptor to modulate the expression of the gene. For example, within 10 kb of DNA surrounding the *D. melanogaster* Eip28/29 gene, 38 sequences were detected with consensus to the EcRE. However, only three of the EcRE consensus sequences were shown to be relevant to the Eip28/29 regulatory control mechanism (Cherbas and Cherbas, 1993).

The physiology of chorion formation

The *A. aegypti* chorion is secreted by the follicular epithelium during the post-trophic phase of egg development. It is assembled from a group of 25-50 kDa glycoproteins, probably chitin and several very high molecular weight proteoglycans (Powell et al, 1988). Although in many species of insects, the chorion shows lamellate formations and crystalline patterns, these are absent in the *A. aegypti* chorion (Mathew and Rai, 1975). The chorion is composed of two layers which are interconnected with pillars and struts, enclosing air spaces (Mathew and Rai, 1975).

The physiological signals that are involved in the formation of the *A. aegypti* chorion are not understood. The mechanism that mediates the switch from vitelline membrane gene expression during the trophic phase of egg development to chorion gene expression during the post trophic stage is also unknown. A hypothetical example of such a switch could be that expression of the *A. aegypti* chorion genes are inhibited by ecdysone. Thus, when the titre of ecdysone falls between 24 and 36 hours following the blood meal, the inhibition of chorion gene expression is lifted. In order to test this regulatory model, it would be useful to obtain molecular probes for chorion gene expression, which are currently not available.
The *Drosophila melanogaster* chorion

The chorion of *D. melanogaster* is a very complex structure composed of an inner layer, perforated with holes and studded with pillars that support an outer layer (Margaritis, 1985). Like the *A. aegypti* chorion, air is contained within the *D. melanogaster* chorionic meshwork. Six major *D. melanogaster* chorion genes (s15, s16, s18, s19, s36 and s38) have been cloned and sequenced (Wong et al. 1985; Levine and Spradling, 1985; Spradling et al. 1987; Fenerjian et al. 1989).

In contrast to *A. aegypti*, the regulation of *D. melanogaster* chorion gene expression has been extensively studied. The proteins that make up the chorion of *D. melanogaster* are secreted in a temporal and spatial pattern that reflects the complex transcriptional regulation of these genes. *D. melanogaster* has been transformed with P-element constructs containing DNA sequences flanking the 5'-ends of several chorion genes, linked to reporter genes (Tolias and Kafatos, 1990). One common feature of all of the chorion gene enhancers is that they all contained the hexameric motif, TCACGT (Tolias and Kafatos, 1990). This motif was also found to be conserved in the promoter regions of four separate chorion genes of *Bombyx mori*, suggesting a highly conserved mechanism of regulatory control (Spoerel et al., 1986).

In one study, various DNA sequences located upstream of the s36 chorion gene TATA box were fused to a heterologous promoter and a reporter gene (hsp70/lacZ). The spatial expression patterns of these constructs following the P-element mediated germline transformation of *D. melanogaster* was examined by observing β-galactosidase activity in dissected ovaries. An 84 bp segment of the proximal 5'-flanking DNA was sufficient to confer wild-type expression to the reporter gene with respect to space and time. Interestingly, when the position of this element was changed with respect to the start site of
transcription, the site (but not timing) of expression within the follicular epithelium was altered (Tolias and Kafatos, 1990).

In studies of the s15 chorion gene promoter region, sufficient regulatory information was found in a 73 bp upstream sequence to permit developmentally correct expression of the gene (Mariani et al., 1988; Romano et al., 1988). This region included three cis-regulatory elements: 1) the essential positive regulatory element, TCACGT (-60 to -55) shared by other chorion genes; 2) a positive element required for the precise "late" expression of this gene 3) a negative element that repressed precocious expression during early choriogenic stages. The proximity of these elements suggested that interactions between the trans- acting factors that bind to these cis-regulatory elements are an integral component of the regulatory mechanism.

In a more detailed study of the s15 chorion gene promoter, gel mobility shift assays were used to identify proteins that bound to the s15 regulatory elements (Shea et al., 1990). Factors that induced shifts were detected in the follicular epithelium at appropriate stages of development. Two of these proteins were isolated and cloned. One of them (CF1), is a member of the steroid hormone receptor superfamily, but was not an ecdysone receptor. The other factor, (CF2) was a novel D. melanogaster member of the C2H2 family of zinc finger transcription factors (Shea et al., 1990).

The silkmoth chorion

There are several differences between the reproductive physiology of the domesticated silkmoth, Bombyx mori, and that of A. aegypti. Nevertheless, the formation of the B. mori chorion is relevant to the study of A. aegypti egg development because the chorion genes are expressed in an ordered succession or developmental profile within the
folicular epithelium. Furthermore, ecdysone has been shown to trigger the complex
program of follicle development including choriogenesis (Tsuchida et al., 1987).

In contrast to A. aegypti, the B. mori ovaries mature in the pupae. Adult silkmoths
mate and lay eggs immediately after emergence. Like D. melanogaster, but unlike A.
aegypti, the B. mori ovaries consist of strings of follicles that are attached to each other in
linear arrays from least mature to ovulated. During the last period of follicle maturation the
epithelial cells that surround the oocyte secrete chorionic proteins. 71 distinct chorion
proteins have been detected by isoelectric focusing, some of which resolve into multiple
bands when fractionated by two-dimensional gel electrophoresis (Nadel and Kafatos,
1980).

The silkmoth chorion has a morphology which is very different from A. aegypti, as
described below by Mazur et al., (1989).

"The basic ultrastructural component (of the silkmoth chorion) is the
fiber. Fibers are arrayed in parallel, within planes or sheets which are
stacked on top of each other. The direction of the fiber axis rotates in
a screw like manner between consecutive planes, from the inner to
the outer surface of the chorion, resulting in a 3 dimensional helicoid.
Within that continuous structure lamellae are perceived as multisheet
layers that correspond to 180° rotation of the fiber axis."

This eggshell structure is not built up layer by layer. Instead, an initial framework is
constructed, which then expands by the insertion of additional fiber sheets (Mazur et al.,
1989).

The B. mori chorion genes are contained in the chorion locus, a large
chromosomal region which contains approximately 200 structural genes. On the basis of
developmental studies, the locus has been subdivided into segments that contain genes that
are expressed by the folicular epithelial cells during early, middle or late developmental
periods of chorion formation (Eickbush and Kafatos, 1982).
Within the chorion locus, the *B. mori* chorion genes are arranged as linked pairs which are divergently transcribed. The genes of each pair border the same 5' flanking sequence. Three distinct 5' flanking sequences were shown to be associated with each of the three distinct developmental classes of genes (Spoerel et al., 1986). Comparisons of the 5' flanking regions, and of the corresponding region of the *D. melanogaster* s-15-1 chorion gene, have revealed numerous sequence elements that are shared. As noted in the previous section of this chapter, one such element (TCACGT) is associated with five sequenced *D. melanogaster* chorion gene promoter regions (Spoerel et al., 1986).

Studies of silkmoth choriogenesis have been advanced by the capacity of vitellogenic follicles to enter and complete the process of choriogenesis in organ culture. Once the *B. mori* choriogenic program becomes established in follicular cells, it can be implemented autonomously in the absence of extra-follicular factors (Swevers and Iatrou, 1992). The chorionic program however, must be established *in vivo*, and cannot be influenced by the addition of ecdysone to the culture medium (Swevers and Iatrou, 1992). These observations led to an investigation of the signaling molecules within the follicular epithelium that coordinate the expression of the chorion genes. One such factor (BCFI) has been identified by Skeiky et al., (1994).

The transcriptional activation of the high cysteine family of chorion coincides with the appearance of a chorion promoter DNA binding factor (BCFI) in follicular cell nuclei. Epithelial cells of vitellogenic and choriogenic follicles that do not express these genes contain high levels of BCFI in their cytoplasm, but not in their nuclei. This transcription factor was shown to migrate from the cytoplasm into the nucleus at the appropriate stage of development. BCFI was shown to belong to the GATA family of zinc finger motif-containing transcription factors by (Drevet et al., 1994).
FIGURE 2.1. Diagrammatic representation of hormone changes from 0 to 6 days after the emergence of the adult female. JH=juvenile hormone, 20-OH=20-hydroxyecdysone. The arrow indicates the time the blood meal was given. The release of EDNH occurs shortly after a blood meal, but its changes in titre are not known. (From Shapiro et al., 1986).
FIGURE 2.2. Model for the control of egg development in *A. aegypti*. Two environmental signals (emergence and the blood meal) and at least three hormones (juvenile hormone (JH), the egg development neurosecretory hormone (EDNH), and 20-hydroxyecdysone) interact to control the development of eggs. Release of JH at emergence induces feeding behavior and mating. JH also stimulates growth of the ovarian follicle to a resting stage, the development of competence of the ovary to respond to EDNH and of the fat body to respond to ecdysone. EDNH released in response to a blood meal stimulates the ovary to produce ecdysone, which is converted to 20-hydroxyecdysone. The latter acts on the fat body, stimulating vitellogenin synthesis. The mature oocytes produce an inhibitory factor that prevents the development of further batches of eggs until another blood meal is taken. (From Hagedorn, 1985).
CHAPTER III

SEQUENCE ANALYSIS OF THREE VITELLINE MEMBRANE GENES IN THE YELLOW FEVER MOSQUITO, \textit{Aedes aegypti}

ABSTRACT

A genomic clone corresponding to a novel \textit{Aedes aegypti} vitelline membrane gene (15a-3) has been isolated. 15a-3 has an open reading frame encoding a peptide of 122 amino acids. The predicted peptide sequence of 15a-3 is similar to those of the \textit{A. aegypti} vitelline membrane genes 15a-1 and 15a-2. Genomic clones corresponding to 15a-1 and 15a-2 were isolated that contain the complete 15a-1 and 15a-2 transcriptional units and contain DNA sequence upstream of the respective coding regions. The deduced amino acid sequences of 15a-1, 15a-2 and 15a-3 contain a region of 45 residues that is highly conserved between the three genes. The conserved region overlaps with a region that is conserved between four \textit{Drosophila melanogaster} vitelline membrane genes. DNA regions of approximately 1.8 kb, 2.1 kb and 2.0 kb were sequenced upstream of the 15a-1, 15a-2 and 15a-3 coding regions respectively. A 360 bp region 5' of the 15a-2 coding region was identified with 72% identity to a region upstream of the \textit{A. aegypti} VgA1 vitellogenin gene. Sequences with similarity to a 20-hydroxyecdysone response element consensus sequence were found upstream of the 15a-1, 15a-2 and 15a-3 coding regions.
INTRODUCTION

The vitelline membrane is the innermost layer of the *Aedes aegypti* eggshell. Genes encoding vitelline membrane proteins have been cloned from the mosquito, *Aedes aegypti* and from *Drosophila melanogaster*. An *A. aegypti* vitelline membrane gene (15a-1) was cloned by differentially screening a genomic library with cDNA prepared from blood-fed and non-blood fed mosquitoes, respectively (Gemmill et al., 1986). Subsequently, a cDNA clone corresponding to 15a-1 and a partial cDNA clone corresponding to a second *A. aegypti* vitelline membrane gene (15a-2) were isolated by Lin et al., (1993). Sequence similarity was detected between 15a-1, 15a-2 and four vitelline membrane genes that have been cloned in *D. melanogaster*. Of these *D. melanogaster* sequences, VM26A1 was reported by Burke et al., (1987), VM26A2 by Popodi et al., (1988), VM32E by Gigliotti et al., (1989) and VM34C by Mindrinos et al., (1985) and Scherer et al., (1993).


A third member of the *A. aegypti* vitelline membrane gene family (15a-3) is reported. DNA fragments upstream of the other two members of this family, 15a-1 and 15a-2 have been sequenced. This facilitates a comparative sequence analysis of members of a superfamily of *A. aegypti* and *D. melanogaster* vitelline membrane genes.
METHODS

Chemicals and cloning vectors

DNA modifying enzymes, nucleotides and digoxigenin-11 dUTP were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Wizard minipreps were from Promega (Madison, WI). Taq polymerase was purchased from BRL Life Technologies (Gaithersburg, MD). $\alpha^{-32}$P dCTP (3000 mCi/ml) was purchased from New England Nuclear (Boston, MA). Other reagents were purchased from Sigma Chemical (St. Louis, MO). pBluescript cloning vector was purchased from Stratagene (La Jolla, CA).

Genomic library screening

The 15a-1 genomic clone was isolated from a Rockefeller strain A. aegypti genomic library in the vector EMBL3 (Stratagene, La Jolla, CA). Construction of the library was described by Barillas-Mury et al., (1993). The genomic clones for 15a-2 and 15a-3 were isolated from a Rockefeller strain A. aegypti genomic library in the vector Lambda-Dash (Stratagene, La Jolla, CA) that was provided by Dr. Anthony James (University of California, Irvine). The hybridization probes corresponding to the 15a-1 and 15a-2 coding regions were labeled with $\alpha^{32}$P dCTP or digoxigenin-11 dUTP using random primers (Feinberg and Vogelstein, 1983). Screening and plaque purification procedures were performed as described by Sambrook et al., (1989).
Restriction fragments of genomic clones 15a-1, 15a-2 and 15a-3 were subcloned into pBluescript II KS\(^{-}\) vector. Sequencing primers were T3, T7 (Stratagene) and several synthetic oligonucleotides based on known sequences that were prepared at the University of Arizona Division of Biotechnology. The double stranded sequencing template was prepared using Promega Wizard Minipreps. Manual sequencing was done by the dideoxy-chain termination method (Sanger et al., 1977) and a Sequenase kit. Sequence was also obtained by an automatic DNA sequencer (Model 373A, Applied Biosystems Int., Forster City, CA) at the Division of Biotechnology at the University of Arizona (Tucson, AZ) and the Molecular Genetics Instrumentation Facility at the University of Georgia (Athens, GA). Complete and overlapping sequences were obtained for each strand thus all sequences were verified by at least one sequencing reaction in the opposite direction (Fig. 3.1).

The sequence data were analyzed using the "Sequence Analysis Software Package" version 7.1 from the Genetics Computer Group (Devereux et al., 1984). GCG programs used included Pileup for multiple sequence alignment and Gap for pairwise comparisons and Blast for database searches. The sequence data were also analyzed using MacVector sequence analysis software (International Biotechnologies).
RESULTS

Selection and identification of genomic clones

To obtain a genomic clone containing DNA sequences upstream of the 15a-1 coding region, approximately 400,000 genomic clones were screened using a radiolabeled 350 bp restriction fragment of a cDNA clone for vitelline membrane gene 15a-1 as a probe (Lin et al., 1993). One positively hybridizing clone was isolated. A 2.1 kb Hind III restriction fragment of the clone that hybridized to the 15a-1 probe was subcloned into pBluescript KS- and sequenced using primers designed for the 3' and 5' termini of the 15a-1 cDNA clone. The sequence of the 15a-1 coding region within the restriction fragment was 100% identical to the previously reported 15a-1 sequence (Lin et al., 1993) and was selected for further study.

To obtain a genomic clone containing DNA sequences upstream of the 15a-2 coding region, approximately 300,000 genomic clones were screened using a radiolabeled 200 bp restriction fragment of the 15a-2 cDNA clone as a probe (Lin et al., 1993). Subsequent restriction enzyme analysis of 7 positively hybridizing clones revealed that one of them corresponded to the previously cloned vitelline membrane gene, 15a-1. Another clone contained a 3.7 kb Eco RI restriction fragment that hybridized with the 15a-2 cDNA probe. A polymerase chain reaction (PCR) using primers designed for the 5' and 3' termini of the 15a-2 partial cDNA clone and the 3.7 kb restriction fragment as template, resulted in DNA product with a molecular weight corresponding to the 15a-2 sequence internal to the primers. The sequence of this fragment was 100% identical to the published 15a-2 sequence (Lin et al., 1993) and was used for further study. Another genomic clone isolated from this screening also contained the 15a-2 coding region.
A 700 bp Cla I restriction fragment that hybridized with the 15a-2 probe was subcloned into pBluescript and the insert was sequenced using T3 and T7 primers. An open reading frame was revealed that was similar to, but distinct from, 15a-1 and 15a-2. This clone will be referred to as 15a-3. Three other genomic clones also contained the 15a-3 gene.

Sequence of the genomic clones

Restriction fragments of the 15a-1, 15a-2 and 15a-3 genomic clones were sequenced. The restriction maps and sequencing strategies are shown in Fig. 3.1. The sequenced restriction fragments included the 15a-1, 15a-2 and 15a-3 coding regions, approximately 1.8, 2.1 and 2.0 kb of upstream sequence, and several hundred base pairs of downstream sequence relative to the respective coding regions. These sequences are shown in Fig. 3.2.

Predicted promoter regions

A comparative approach was used to predict the promoter elements upstream of the 15a-1, 15a-2 and 15a-3 coding regions. Sequences consistent with the most frequently occurring arthropod initiator site (Cherbas and Cherbas, 1993) were identified in 15a-1, 15a-2 and 15a-3 (Fig. 3.3). Predicted TATA boxes (TATAAA) were identified 27, 26 and 26 bp upstream from the respective 15a-1, 15a-2 and 15a-3 predicted initiator sequences (Fig. 3.3). The spacing between the capsite and TATA box is expected on the basis of consensus with other eukaryotic promoters (Breathnach and Chambon, 1981). In-frame ATG sequences were identified 65, 55 and 76 bp downstream from the respective 15a-1, 15a-2 and 15a-3 arthropod initiator sequences (Fig. 3.3). The ATG sequences are likely candidates for the translational initiation sites because they are the first ATG sequences
following the predicted 5' end of the mRNA, and the surrounding nucleotides contain the highly conserved adenosine residue at position -3, (Kozak, 1986; Cavener and Ray, 1987).

**Analysis of the predicted 15a-3 transcriptional unit**

The predicted 15a-3 transcriptional unit contains an 83 base 5'- noncoding region, 366 bp open reading frame and a 137 bp predicted 3'- untranslated region. The predicted 15a-3 peptide sequence is shown in Fig. 3.2. No introns are predicted in the 15a-3 sequence which is consistent with *A. aegypti* vitelline membrane genes 15a-1, 15a-2 and the vitelline membrane genes of *D. melanogaster*. A stop codon (UAA) consistent with the consensus stop codon for *D. melanogaster* and other invertebrates (Cavener and Ray, 1991) was located 403 bp following the predicted transcriptional initiation site (3. 2). This was followed by a poly-A addition signal consensus sequence (AATAAA) (Proudfoot and Brownlee, 1976; Zarkower and Wickens, 1987) beginning at 588 bp following the predicted transcriptional initiation site (Fig. 3. 2).

The open reading frame in the 15a-3 genomic sequence encodes a predicted protein of 122 amino acids. Hydropathy analysis (Fig. 3.4) shows that the first 18 amino acids of the peptide are hydrophobic. They are followed by a predicted signal cleavage site, using the -3, -1 rule of the signal peptide cleavage site of Von Heijne (1990). This suggests that the 15a-3 protein is synthesized with a signal peptide allowing for secretion. Following signal peptide cleavage, the mature 15a-3 peptide would be 11.1 kDa. The peptide sequence of the 15a-3 was compared by multiple sequence alignment to those of 15a-1 and 15a-2 (Fig. 3.5). A series of 8 proline residues that is present from positions 24 to 31 in the 15a-3 peptide sequence is not contained in the 15a-1 or 15a-2 sequences.
A conserved region of 45 amino acids previously noted in 15a-1 and 15a-2 (Lin et al., 1993) is also present from residues 53 to 948 in 15a-3 (Fig. 3.6). The 15a-3 conserved region is highlighted in the hydrophilicity plot of the predicted peptide sequence (Fig. 3.4). The conserved region overlaps with a conserved region that has been described within three *D. melanogaster* vitelline membrane genes (VM26A1, VM26A2 and VM34C) by Scherer et al., (1988) and the *D. melanogaster* vitelline membrane gene VM32E (Gigliotti et al., 1989). A multiple sequence alignment of the *A. aegypti* and *D. melanogaster* conserved regions is shown in Fig. 3.6.

**Potential ecdysone responsive elements**

The upstream regions of 15a-1, 15a-2 and 15a-3 were analyzed for the presence of *D. melanogaster* ecdysone responsive element (DmEcRE) consensus sequences. The sequence \[\text{PuG(G/T)T(C/G)A(N)TG(C/A)(C/A)(C/t)Py}\] has been empirically shown to contain residues that are critical for the binding of *D. melanogaster* ecdysone-receptors to oligonucleotides in a gel mobility shift assay (Antoniewski et al., 1993). Several sequences with partial similarity to the DmEcRE consensus sequence were identified upstream of the 15a-1, 15a-2 and 15a-3 coding regions (Table 3.1).
DISCUSSION

15a-3 is a vitelline membrane gene

The 15a-3 vitelline membrane gene was identified by sequence analysis of a genomic clone that hybridized with a 15a-2 probe. The 15a-3 genomic clone contains an open reading frame that is similar to 15a-1 and 15a-2. This similarity is apparent upon inspection of a multiple sequence alignment analysis of the 15a-1, 15a-2 and 15a-3 predicted peptide sequences (Fig. 3.5). The percentage of similarity between the three A. aegypti vitelline membrane protein sequences was determined using the GCG Gap program allowing for a gap penalty of 3, as shown below.

<table>
<thead>
<tr>
<th></th>
<th>15a-2</th>
<th>15a-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>15a-1</td>
<td>84.3</td>
<td>91.7</td>
</tr>
<tr>
<td>15a-2</td>
<td>85.3</td>
<td></td>
</tr>
</tbody>
</table>

The statistical significance of the comparisons between the A. aegypti vitelline membrane genes was determined calculating an adjusted quality (Q') of the comparison by the formula of Barker and Dayhoff (1972) which is shown below. The adjusted quality is an index of the probability that the quality of the tested comparison could be obtained by random chance. The probability of obtaining the adjusted score can be determined from a z-score table. For example, the probability of obtaining a score of 3 is 0.13%. An adjusted score of 3.0 or greater is required for a reasonable confidence that two sequences are significantly related (Barker and Dayhoff, 1972; Gribisow and Burgess, 1986; Romans et al., 1995). Quality scores (Q) were calculated by the GCG Gap program, allowing for a gap penalty of 3.

\[
Q' = \frac{Q - \text{Mean} Q \text{ of 100 random comparisons}}{\text{Standard deviation of 100 random comparisons}}
\]
The adjusted qualities of the comparisons between the 15a-1, 15a-2 and 15a-3 genes and between the *A. aegypti* vitelline membrane genes are shown below. These are all greater than three, thus the sequences are significantly similar to each other.

<table>
<thead>
<tr>
<th></th>
<th>15a-2</th>
<th>15a-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>15a-1</td>
<td>12.2</td>
<td>22.3</td>
</tr>
<tr>
<td>15a-2</td>
<td>20.3</td>
<td></td>
</tr>
</tbody>
</table>

**Conserved regions**

The peptide sequence from residues 61 and 98 of the 15a-3 sequence is highly conserved between the 15a-1, 15a-2 and 15a-3 peptide sequences (Fig. 3.6). The *D. melanogaster* vitelline membrane genes contain a conserved region of 114 nucleotides which was described in VM26A1, VM26A2 and VM34C by Scherer *et al.*, (1988) and in VM32E by Gigliotti *et al.*, (1989). The *D. melanogaster* conserved region overlaps in sequence with the *A. aegypti* conserved region (Fig. 3.6).

It is possible that the conserved regions of the vitelline membrane genes provide a structural conformation that is favorable to the formation of cystine bridges. All of the cysteine residues in the *A. aegypti* and *D. melanogaster* vitelline membrane sequences are located within the conserved region (Fig. 3.6) with the exception of a cysteine at position 3 in the mature VM32E peptide (Gigliotti *et al.*, 1989). These residues are conserved between the *A. aegypti* and *D. melanogaster* vitelline membrane genes in the respective conserved regions (Fig. 3.6). Evidence for the formation of cysteine bridges between *D. melanogaster* vitelline membrane proteins was provided by Petri *et al.*, (1979), who showed that vitelline membrane proteins are insoluble in 2% SDS but soluble in a combination of 2% SDS and β-merceptoethanol.
Proline content

The *A. aegypti* vitelline membrane genes have an unusually high proline content, when compared to the proline content of the *D. melanogaster* vitelline membrane proteins. The average proline content of the *A. aegypti* vitelline membrane genes is about 30%. This is approximately double the average proline content of the *D. melanogaster* vitelline membrane genes, which is about 15%. The average proline content of 314 representative proteins is approximately 5% (Dayhoff *et al.*, 1979).

Another unusual feature of the *A. aegypti* vitelline membrane genes is that they contain at least one sequence of 6-8 consecutive proline residues. For example, 15a-3 contains two stretches of 5 proline residues and one stretch of 8 proline residues. In contrast, there are no more than two consecutive prolines in any of the *D. melanogaster* sequences. Computer assisted searches of known protein sequences using Blast and other programs in the GCG software package shows that stretches of 6 or more consecutive proline residues are rare among the mature peptides found in invertebrates.

Analysis of the of upstream regions

Regulatory elements have been analyzed upstream of three *D. melanogaster* vitelline membrane genes. Savant and Waring (1989) demonstrated that all of the necessary cis-acting regulatory elements for wild type expression were located within 147 bp of the VM26A2 coding region. Jin and Petri (1993) demonstrated that 597 bp of 5' flanking DNA is sufficient to promote expression of a lacZ reporter gene with the appropriate tissue and stage specificities. Gargiulo *et al.*, (1991) showed that the first 465 nucleotides upstream of VM32E confer the appropriate stage and tissue specific expression to a reporter gene.
A comparative analysis of three *D. melanogaster* vitelline membrane upstream sequences (VM26A1, VM34C and VM32E) revealed a heptameric sequence (AAGTGC) (Scherer et al., 1993). This consensus sequence is also present upstream of the *D. melanogaster* yolk proteins, YP1-3. All of these regions confer ovarian temporal and tissue-specific expression to their respective transcriptional units. The significance of this heptameric sequence has not yet been determined by any functional assay.

A comparative analysis of the upstream regions of the 15a-1, 15a-2 and 15a-3 vitelline membrane genes was performed using the GCG programs Bestfit and Gap. No significant similarity was observed between the 15a-1, 15a-2 and 15a-3 sequences upstream of the predicted TATA boxes. Furthermore, no significant similarity was detected between the upstream regions of the *A. aegypti* 15a-1, 15a-2 and 15a-3 vitelline membrane genes and sequences upstream of the VM26A1, VM34C and VM32E coding regions. Complete consensus sequences corresponding to the *D. melanogaster* heptamer sequence (Scherer et al, 1993) were not found.

There is a region of similarity between the 15a-2 upstream sequence and that of an *A. aegypti* vitellogenin gene, VgA1 (Romans et al., 1995). This 360 bp region is 810 bp upstream of the 15a-2 transcriptional start site and is 72% identical to a region 750 bp upstream of the VgA1 transcriptional start site (Fig. 3.7). Both sequences contain a partial consensus sequence for the ecdysone responsive element (Antoniewski et al., 1993). The timing of VgA1 expression is similar to 15a-2 expression. However the site of VgA1 expression is the fat body rather than the ovaries (Gemmill et al., 1986; Lin et al., 1993). Like 15a-2, the expression of the vitellogenin VgA1 gene is regulated by ecdysone (Racioppi et al., 1986). Ecdysone probably acts on VgA1 expression via a protein intermediate (unpublished observations; Dietsch et al., 1995).
Potential ecdysone response elements

The formation of the *A. aegypti* vitelline membrane coincides with a sharp rise in ecdysone titer following the blood meal (Hagedorn *et al.*, 1975; Racioppi *et al.*, 1986, Lin *et al.*, 1993). Raikhel and Lea (1991) have shown that formation of the vitelline membrane (envelope) can be induced by two 500 pg injections of ecdysone into decapitated females that were given blood by enema. Lin *et al.*, (1993) demonstrated by northern analysis that the 15a-1 vitelline membrane gene was expressed in ovaries of blood-fed and decapitated females in response to an injection of $1 \mu g$ of ecdysone. In addition, 15a-1 message was detected in ovaries that were cultured in medium containing $10^{-5} M$ ecdysone, but not in ovaries that were cultured in medium without ecdysone (Lin *et al.*, 1993).

An *A. aegypti* ecdysone receptor (AaEcR) has been cloned by Cho *et al.*, (1995). The DNA sequence of the DNA binding domain of the AaEcR is 97% identical to the DNA binding domain of the *D. melanogaster* ecdysone receptor. Thus it is likely that the DmEcRE (Antoniewski *et al.*, 1993) is conserved in *A. aegypti*. In this case, the consensus sequences for the DmEcRE that were found upstream of the 15a-1, 15a-2 and 15a-3 (Table 3.1) gene may interact with an AaEcR. Further analysis is required to determine whether the ecdysone responsive elements upstream of the *A. aegypti* vitelline membrane gene coding regions are involved in the regulation of these genes.

*Acknowledgments* - This work was supported by NIH grant (HD24869) to Drs. Henry H. Hagedorn and Ann M. Fallon, and by the John D. and Catherine T. MacArthur Foundation. The author thanks Dr. Anthony James at the University of California, Irvine, for the Lambda-Dash *A. aegypti* genomic library and Skip Vaught at the University of Arizona Division of Biotechnology for sequencing the majority of the DNA reported.
TABLE 3.1. Potential ecdysone responsive elements (EcREs) upstream of 15a-1, 15a-2 and 15a-3. These EcREs are also shown as double underlined sequences in Fig. 3.2. Plus strand is defined as the 5' to 3' orientation with respect to the coding region. The ecdysone consensus sequence is defined as PuG(G/T)T(C/A)A(N)TG(C/A)(C/A)Py (Antoniewski et al., 1993).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Strand</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>15a-1</td>
<td>-1214</td>
<td>minus</td>
<td>GGGTCACccGaATT</td>
</tr>
<tr>
<td>15a-2</td>
<td>-1941</td>
<td>plus</td>
<td>GGGgGACccGACCT</td>
</tr>
<tr>
<td></td>
<td>-766</td>
<td>plus</td>
<td>AGTTGAGTGtCCg</td>
</tr>
<tr>
<td>15a-3</td>
<td>-1669</td>
<td>minus</td>
<td>GGTgCATTGAtTT</td>
</tr>
<tr>
<td></td>
<td>-367</td>
<td>plus</td>
<td>AGTTCcGTGCACT</td>
</tr>
<tr>
<td></td>
<td>+62</td>
<td>minus</td>
<td>AGTTGgATGgATT</td>
</tr>
</tbody>
</table>
FIGURE 3.1. Restriction maps and sequencing strategy of the 15a-1, 2 and 3 genomic clones. Restriction endonuclease designations are B, Bst X1; E, Eco RI; C, Cla I; H, HindIII; K, Kpn I; S, Sac I; X, Xba I. Closed and open rectangles represent, respectively, the predicted signal sequences and coding region of the predicted protein. The arrows indicate the direction and length of individual sequencing reactions.
a) 15a-1

-1765 aagctttcattccataaaaaagattggaatacgggtgagctatcattccaagaatttagatattctcaaaaa
-1698 aaaaaaaaaagtctaatgtacctcatcagtggtgcagatccaaaaaatatgtactgttacttttttttttcttttctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
FIGURE 3.2. Nucleotide sequences within three *A. aegypti* vitelline membrane clones a) 15a-1 b) 15a-2 and c) 15a-3. Single underlined regions are, in order, the TATA box, the transcription start site, the first ATG, the stop codon, and the poly-A addition signal. Dotted underlined regions indicate the predicted signal peptides. Double underlines indicate potential ecdysone responsive elements. Nucleotides are numbered at the left, in reference to the transcription start site. Amino acids are also marked at the left. The stop codon (TAA) is marked with an asterisk.


<table>
<thead>
<tr>
<th></th>
<th>TATA box</th>
<th>Arthropod Initiator</th>
</tr>
</thead>
<tbody>
<tr>
<td>15a-1 -33</td>
<td><code>tataaaacgccagcctccagccagacttccacatcagttacacc..</code></td>
<td></td>
</tr>
<tr>
<td>15a-2 -32</td>
<td><code>tataaaatacctccagccggtactcccatcagttacaccac</code></td>
<td></td>
</tr>
<tr>
<td>15a-3 -32</td>
<td><code>tataaaacctttatcagttacaccac</code></td>
<td></td>
</tr>
<tr>
<td>15a-1 +10</td>
<td><code>gcaacttgcccaacagtgtactacagtcccaccgaggaattcccat</code></td>
<td></td>
</tr>
<tr>
<td>15a-2 +13</td>
<td><code>caagcatccacctccggtgttacacatctaaatcaacagcaaatctaa</code></td>
<td></td>
</tr>
<tr>
<td>15a-3 +13</td>
<td><code>acagtatcagaacatgtttacatgatattttccacgacctccgaagga</code></td>
<td></td>
</tr>
<tr>
<td>15a-1 +56</td>
<td><code>cccaactcagtaaccatg</code></td>
<td></td>
</tr>
<tr>
<td>15a-2 +59</td>
<td><code>caatg</code></td>
<td></td>
</tr>
<tr>
<td>15a-3 +59</td>
<td><code>atccatccacctttaccggaaccatg</code></td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 3.3.** Promoter regions of 15a-1, 15a-2 and 15a-3 showing conserved TATA box and arthropod initiator sequences in bold. Conserved nucleotides in 15a-1 and 15a-2 surrounding the initiation site are underlined. The translational start site (ATG) is shown in bold.
FIGURE 3.4 Hydropathy analysis of the deduced 15a-3 vitelline membrane peptide sequence according to the method of Kyte and Doolittle (1982). The plot was made using the MacVector (IBI) sequence analysis software. A window size of 7 was used. The conserved region is highlighted in a box.
FIGURE 3.5. Multiple sequence alignment of the predicted mature peptide sequences (using CGC Pileup after removal of the predicted signal peptides, gap creation penalty = 3.0) from the 15a-1, 15a-2 and 15a-3 genes of *A. aegypti*. Residue numbers beginning from the predicted start codons are listed at the beginning of each line. Gaps that improve the alignment are shown as spaces. The consensus sequence is determined by identities between two or more sequences in the multiple sequence alignment.
<p>| | | | | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>15a-1</td>
<td>36</td>
<td>HAHHPAPAPVVHTFVHAPHAKCGANLLVGCAAPSVAVHPVPCVP1pGH</td>
<td>81</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15a-2</td>
<td>45</td>
<td>HHAppplhPVVHTFYVAPAPaAKCGANLLVGCAAPSVAVHPVPCVPVHpH</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15a-3</td>
<td>53</td>
<td>HHAHPAPAPVVHTFVHAPHAKCGANLLVGCAAPSVAVHPVPCVPVHG</td>
<td>98</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VM26A</td>
<td>70</td>
<td>SIPAPPCPKNYLFSCQPLAPVPCASPAPSYGSAGAY</td>
<td>108</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VM26A2</td>
<td>116</td>
<td>SIPsPPCPKNYLFSCQPlqPVP1sAPAqSYGSAGAY</td>
<td>154</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VM34C</td>
<td>68</td>
<td>SIPAPPCPKNYLFSCQPNLAAPVPCASPAPSYGSAGAY</td>
<td>106</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VM32E</td>
<td>36</td>
<td>gyPAPPCPcNYLFSCQPNLAAPCaqeAPaYGSAGAYt</td>
<td>74</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 3.6.** Alignment of the protein sequences of the conserved regions in the *A. aegypti* and *D. melanogaster* vitelline membrane genes. Conserved residues are shown in upper case letters. Residues that are conserved between the *A. aegypti* and *D. melanogaster* are indicated with a bar. Conserved cystine residues are marked with an asterisk. Residues are numbered in reference to the translational start site as +1.
FIGURE 3.7. Similar region contained in both 15a-2 and A. aegypti vitellogenin gene VgAl upstream regions. Using the GCG program Bestfit with a gap creation penalty of 5.0, a 72% identity was observed within a 360 bp region upstream of the 15a-l coding region and upstream of the A. aegypti vitellogenin gene VgAl (Romans et al., 1995). The regions were inverted in orientation between the two sequences and started at -1110 and -1180 bp upstream of the 15a-2 and VgAl coding regions, respectively.
CHAPTER IV

REGULATION OF VITELLINE MEMBRANE GENE EXPRESSION IN THE YELLOW FEVER MOSQUITO, Aedes aegypti

ABSTRACT

The transcriptional regulation of three Aedes aegypti vitelline membrane genes, 15a-1, 15a-2 and 15a-3 was examined. The respective timing and sites of expression of the three transcripts were determined by northern blot analysis and whole-mount in situ hybridization using mutually exclusive probes. The temporal pattern of 15a-1, 15a-2 and 15a-3 expression was similar. The spatial pattern of expression differed between the three genes. Only 15a-2 was expressed at the anterior region in addition to the remainder of the follicle. 15a-1 and 15a-3 were only expressed in the mid and posterior regions of the follicle. The effect of 10^{-5} M 20-hydroxyecdysone on vitelline membrane gene expression was examined in vitro. Expression of 15a-1 was lower in ovaries that were dissected at 10, 24 and 36 h after the blood meal and incubated for 10 h in culture medium, as compared to ovaries that were analyzed immediately after dissection at these intervals. Expression of 15a-1 was higher in ovaries that were dissected at intervals of 0, 2, 10 and 24 h after a blood meal and were cultured in medium containing 20-hydroxyecdysone, as compared to control incubations. In ovaries that were dissected at 36 h after a blood meal, incubation in 20-hydroxyecdysone had no effect on 15a-1 expression, as compared to control incubations.
INTRODUCTION

The vitelline membrane is the innermost layer of the mosquito eggshell. Vitelline membrane proteins are secreted by the follicular epithelium. The formation and ultrastructure of the \textit{A. aegypti} vitelline membrane has been studied by Anderson and Spielman (1971 and 1973), Mathew and Rai (1975), Powell et al., (1988) and Raikhel and Lea (1982 and 1991). The formation of the \textit{A. aegypti} vitelline membrane can be experimentally induced by injections of 20-hydroxyecdysone (ecdysone) (Raikhel and Lea, 1982 and 1991).

An \textit{A. aegypti} vitelline membrane gene (15a-1) was cloned by differentially screening a genomic library (Gemmill \textit{et al.}, 1986). A related vitelline membrane cDNA clone (15a-2) was identified by Lin \textit{et al.}, (1993). A third vitelline membrane gene (15a-3) was cloned and sequenced by Edwards and Hagedorn (unpublished).

Four vitelline membrane genes have been cloned and sequenced in \textit{Drosophila melanogaster}. These are, according to the nomenclature of Jin and Petri (1994), VM26A1 (Burke \textit{et al.}, 1987), VM26A2 (Popodi \textit{et al.}, 1988), VM32E (Gigliotti \textit{et al.}, 1989) and VM34C (Mindrinos et al., 1985). The \textit{D. melanogaster} vitelline membrane genes are related to the vitelline membrane genes of \textit{A. aegypti} (Lin \textit{et al}, 1993).

The sites and timing of 15a-1, 15a-2 and 15a-3 expression were examined order to explore the possibility that these genes share a conserved regulatory control mechanism. The role of ecdysone in vitelline membrane gene expression was analyzed \textit{in vitro}. These experiments were performed at increasing intervals following a blood meal.
METHODS

Animals

The Rockefeller strain of *A. aegypti* was used in all of the experiments and mosquitoes were reared as described by Shapiro and Hagedorn, (1982). Adult females 4 days after eclosion were fed on warmed cow blood supplemented with 2 mg/ml isoleucine and 1mM ATP through a stretched Parafilm membrane.

Chemicals

DNA modifying enzymes, nucleotides, digoxigenin-11 dUTP and proteinase K were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Taq polymerase was purchased from BRL Life Technologies (Gaithersburg, MD). α–32P dCTP (3000 Ci/mmol) was purchased from New England Nuclear (Boston, Mass). Hybond N+ nylon membranes were purchased from Amersham (Arlington Heights, Ill). Paraformaldehyde and formaldehyde were purchased from Polysciences (Warrington, PA). 20-hydroxyecdysone was purchased from Sigma (St. Louis, MO). The RNaid RNA purification kit was purchased from Bio101 (La Jolla, CA). pBluescript cloning vector was purchased from Stratagene (La Jolla, CA).

Hybridization probes

For whole-mount *in situ* hybridization experiments, mutually exclusive probes were constructed using the polymerase chain reaction (PCR). Antisense (3' to 5'-orientation) and sense (5' to 3'-orientation) primers were designed based on the predicted 3'-untranslated regions of the 15a-1, 15a-2 and 15a-3 transcriptional units respectively (Fig. 4.1). Using restriction fragments containing the 3'-untranslated regions of 15a-1, 15a-2
and 15a-3 as templates, single-stranded probes were generated using PCR. PCR reactions were carried out in a Coy Model 50 TempCycler (Ann Arbor, MI). The reaction mixtures contained 1X PCR buffer (Life Technologies), 1.6 mM MgCl$_2$, 0.1mM dATP, dCTP and dGTP, 0.035mM dTTP and 0.065 mM Digoxigenin-11 dUTP, 0.5μM of a single primer and 1.25 units of Taq polymerase. Taq polymerase was added at 75 °C. Probe concentration was estimated by colorometric detection in comparison to digoxigenin-labeled control DNA (Boehringer Mannheim).

For northern analysis of the relative timing 15a-1, 15a-2 and 15a-3 gene expression, mutually exclusive hybridization probes were used. The probes of 200, 160 and 130 bp, respectively, were prepared by extension from specific primers. The primers were constructed in the antisense orientation and corresponded to the 3'- end of the 3'-untranslated regions of 15a-1, 15a-2 and 15a-3 (Fig. 4.1). Reactions were performed using α$_{32}$P dCTP (3000 mCi / ml) and the Klenow fragment of DNA polymerase I, using restriction fragments containing the respective 3'-untranslated regions as template. For northern blot analysis of the effect of ecdysone on ovaries cultured in vitro, a 350 bp restriction fragment of the 15a-1 coding region was labeled with α$_{32}$P dCTP using random primers (Feinberg and Vogelstein, 1983).

**RNA isolation and characterization**

Ovaries were dissected from female mosquitoes at intervals following a blood meal. Development of ovaries was determined by measuring yolk length using the ocular micrometer on a dissecting microscope. Yolk lengths were compared to published data of Yonge and Hagedorn (1977) and Raikhel and Lea (1991). Total RNA was prepared from dissected ovaries using an RNaid RNA purification kit (Bio 101) following the manufacturers specifications and modifications of Noriega and Wells (1993).
The concentration of total RNA samples was determined by spectrophotometry. For the time course experiment, approximately 4 µg of total RNA were loaded per lane. The relative amounts of mRNA loaded in the time course experiment were also estimated using an A. aegypti actin gene probe, provided by Dr. Elizabeth Keller (Cornell University) and an Aedes albopictus 18S ribosomal RNA probe (18S RNA), provided by Dr. Ann M. Fallon (University of Minnesota).

For in vitro culture experiments, 10 to 12 µg of total RNA were loaded per lane for all time points except for the 24 and 36 hour time points, in which 1.2 µg of RNA were loaded per lane. Total RNA was separated on a 1.5% agarose gel containing 10% formaldehyde and transferred to a charged nylon membrane (Hybond N+, Ammersham). Transfer of RNA was performed using a VacuGene XL vacuum blotting system (Pharmacia Biotech, Uppsala, Sweden). RNA was crosslinked to the nylon membrane using a UV crosslinker (Stratalinker, Stratagene). Hybridizations were performed at 65 ºC in 5X SSPE, 5X Denhard's solution, 0.1 mg/ ml sheared salmon sperm and 0.1% SDS.

The amount of radioactivity bound to individual samples on the nylon membrane was determined using a Betascope (Betagene, Waltham, MA). Bound radioactivity was removed by several washes in 5% SDS at 100 ºC. The effectiveness of the removal was determined using the Betascope. In the in vitro culture experiments, CPM values were used to determine the ratio of 15a-1 to 18S RNA signals. The ratio data were analyzed because they reduced sample variation due to differences in sample loading, effectiveness of blotting and hybridization conditions.

**Whole-mount In situ hybridization**
Ovaries were dissected from mosquitoes following a blood meal and fixed overnight in freshly prepared 4% paraformaldehyde in 1X PBS containing 0.1% Tween-20 (PBT). Ovaries were dehydrated through a methanol-PBT series (25%, 50% and 75% methanol in PBT and 100% methanol) and transferred to 100% ethanol for storage at -20 °C. Ovaries were cleared in 100% xylene for two hours after passage through a series of washes in 25% and 50% xylene in ethanol. Xylene treatment was followed by washes in 50% xylene in ethanol and finally 100% ethanol. Ovaries were rehydrated through a methanol-PBT with 5% formaldehyde series, and rinsed in PBT. Ovaries were permeablized by treatment for 30 minutes in PBT plus 50 µg/ml proteinase K. Protease treatment was stopped by washing with PBT plus 2 mg/ml glycine. Post-fixation was performed in 5% formaldehyde in PBT.

Ovaries that were used in a single experiment were fixed, cleared and permeablized with proteinase K together in order to reduce variations in background and signal intensity. Subsequently, the ovaries were divided into groups and hybridized separately with digoxigenin-labeled mutually exclusive probes. Hybridization, washing and detection steps were performed according to the methods of Tautz and Pfeiffle (1989). Following color development, ovaries were mounted in 50% glycerol containing 10mM Tris pH 7.4 and 1mM EDTA and photographed using a compound microscope and Kodak Ektachrome 160 Tungsten film or Ilford ASA 50 black and white film.

**In vitro culture of ovaries**

Ten pairs of ovaries were dissected from mosquitoes at intervals (0, 2, 10, 24 and 36 h) following a blood meal. Mosquitoes at 0 h PBM were collected as they finished feeding. Ovaries were cultured for 10 h at 26 °C in a complex medium formulated for *A.*
*Aegypti* organ culture (Bohm et al., 1978). For ecdysone treatments, the culture medium was brought to a final concentration of $10^{-5}$ M 20-hydroxyecdysone (Sigma).

Following hybridization with the 15a-1 probe, northern blots were hybridized with an 18S RNA probe. The 18S RNA CPM values were used as an estimate of the relative amounts of total RNA that were loaded per lane. These values were used to determine the relative 15a-1 signal by calculating the ratio of the 15a-1 CPM values to the 18S RNA CPM values. In experiments that were performed with at least three replicates, differences between the mean ratio values were analyzed using the Student's t-test. The mean ratio values of all of the experiments performed at each PBM interval were analyzed by nested ANOVA.
RESULTS

Timing of expression

The relative timing of 15a-1, 15a-2 and 15a-3 expression following a blood meal was determined by northern blot analysis. Due to the highly conserved coding regions of the *A. aegypti* vitelline membrane genes, it was necessary to use mutually exclusive probes. These probes were designed from 3’- untranslated regions of the three vitelline membrane genes which contained no sequence similarity (Fig. 4.1).

Northern blots containing samples of total ovarian RNA prepared at 5 hour intervals from 0 to 60 h PBM were sequentially hybridized with mutually exclusive probes for 15a-1, 15a-2, 15a-3 as well as actin and *A. albopictus* 18S ribosomal RNA (18S RNA) probes (Figs 4.2 and 4.3). The counts per minute (CPM) values corresponding to these probes are shown in Table 4.1.

Expression of 15a-1, 15a-2 and 15a-3 was first detected from 5 to 7.5 h PBM. The highest level of expression of the three genes was observed from 30 to 45 h PBM. By 60 h PBM, expression was not detected. The CPM values for 15a-1, 15a-2 and 15a-3 relative to the maximal values for expression at each time interval are shown in Fig. 4.2. No major differences were detected between the relative temporal expression profiles of 15a-1, 15a-2 or 15a-3.

Sites of expression

The relative sites of 15a-1, 15a-2 and 15a-3 expression were determined by whole mount *in situ* hybridization using digoxigen-labeled probes. As with the time course northern blot experiment, mutually exclusive probes corresponding to the 3’- untranslated regions of the three vitelline membrane genes were used. Groups of ovaries were
dissected from mosquitoes at either 24 or 36 h PBM. The ovaries were hybridized separately with the respective probes.

The relative sites of expression of 15a-1, 15a-2 and 15a-3 at 24 h and 36 h PBM are shown in Figs. 4.4a - 4.4f. The site of 15a-2 expression was strikingly different from those of 15a-1 and 15a-3. Only 15a-2 was consistently expressed in the anterior region, in addition to the remainder of the follicle (Figs. 4.4 a, c, f). By 36 h PBM, 15a-2 expression was especially robust in a set of 6-8 follicle cells at the anterior pole (Fig. 4.4 f). Both 15a-1 and 15a-3 were expressed in mid and posterior regions of the follicle (Fig. 4.4 b, d), although a very low level of 15a-1 expression was occasionally observed at the anterior pole at 36 h PBM (Fig. 4.4 e).

No 15a-3 message was detected in ovaries that were dissected from non-blood fed mosquitoes. Only weak signal was detected with the 15a-3 probe in ovaries that were dissected from mosquitoes 10 h and 50 h PBM (unpublished observations). No signal was detected in ovaries dissected from mosquitoes at either 24 or 36 h PBM using 15a-1, 15a-2 or 15a-3 hybridization probes that were prepared by primer extension in the sense orientation as a control (unpublished observations).

Effect of in vitro culture

Northern blot analysis was used to determine the effect of in vitro culture on vitelline membrane gene expression. In each experiment, ovaries were dissected at intervals of either 0, 2, 10, 24 or 36 h PBM. In the groups that were not incubated in medium (No Incubation), total RNA was extracted from 10 ovaries per replicate immediately after dissection. In groups that were incubated in vitro without ecdysone (Control Incubation), 10 ovaries were dissected and incubated for 10 h in culture medium
that did not contain ecdysone. In the incubated ovary groups, total RNA was extracted from the ovaries immediately following their in vitro culture.

The coding region of 15a-1 was used as a hybridization probe in these experiments. This probe cross reacts with other A. aegypti vitelline membrane genes, resulting in a higher signal than any of the mutually exclusive probes. Further study will be required to determine whether the A. aegypti vitelline membrane genes differ in their response to ecdysone in vitro. A composite figure showing representative northern blot results at each time point is shown in Fig. 4.5.

In ovaries dissected at 10 h PBM, vitelline membrane message expression was lower in the incubated ovaries than in ovaries that were analyzed immediately after dissection (P= 0.0015) (Table 4.2). This trend was observed at 24 h PBM and at 36 h PBM (Table 4.2). No differences were detected at 0 or 2 h PBM, since vitelline membrane gene expression was either absent or very low at these intervals.

**Effect of ecdysone in vitro**

The effect of ecdysone on vitelline membrane gene expression was also determined by northern analysis. In each experiment, ovaries were dissected at intervals of either 0, 2, 10, 24 or 36 h PBM. Results from incubated control ovaries are described above. In experimental groups in which the effect of ecdysone was tested (Ecdysone Incubation), 10 ovaries per replicate were dissected and then incubated for 10 h in culture medium containing 10^{-5} M ecdysone. In both groups, total RNA was extracted from the ovaries immediately following their in vitro culture and used for northern analysis.

From 0 to 24 h PBM, message expression was higher in ovaries that were cultured in ecdysone, as compared to ovaries that were cultured in medium that did not contain ecdysone. At 0 h PBM, the expression of messages that hybridized with the 15a-1 probe
was only detected in ovaries incubated in ecdysone (Table 4.2). At 2 h PBM, values that were observed in ecdysone treated ovaries were significantly higher than those observed in the control groups ($P=0.0006$) (Table 4.2). At 10 h PBM, values that were observed in ovaries that were incubated in ecdysone were significantly higher than those observed in control groups ($P=0.0031$) (Table 4.2). At 36 h PBM, no significant difference was detected between message expression in ovaries incubated with or without ecdysone (Table 4.2). This trend was observed in at least 3 separate experiments. These data suggest that ecdysone incubation did not elevate the expression of vitelline membrane genes in ovaries that were dissected 36 h PBM.
DISCUSSION

Timing of expression

The relative timing of 15a-1, 15a-2 and 15a-3 expression following a blood meal was determined by northern analysis (Figs. 4.2 and 4.3). Expression was observed from 5 to 55 h PBM, with a broad peak of expression between 30 and 45 h PBM. The expression profiles of the three genes were very similar (Fig. 4.2). Since there were no major differences between the temporal profiles of 15a-1, 15a-2 and 15a-3 expression, it is possible that they share a common regulatory mechanism with respect to their timing.

Similar time frames of vitelline membrane gene expression were observed in three separate experiments (unpublished observations). These expression profiles were not consistent with observations that the formation of the vitelline membrane is complete at 36 h PBM (Raikhel and Lea, 1991). They were more consistent with the observations of Anderson and Spielman (1973), that formation of the vitelline membrane was complete 50 to 60 h PBM (in mosquitoes that were kept at 22 °C). These discrepancies in timing are likely due to differences in the rates of egg development in the respective experiments.

Spatial expression

The spatial expression of 15a-1, 15a-2 and 15a-3 was not coordinate (Fig. 4.4). Only 15a-2 was consistently expressed in the anterior region of the follicle, whereas all three genes were expressed in the remainder of the follicle. At least in the anterior region of the follicle, 15a-1, 15a-2 and 15a-3 do not share a common regulatory mechanism with respect to their spatial expression.

In A. aegypti, plaques of vitelline membrane proteins were not found in the anterior region of the follicle during the period of vitelline membrane formation (Anderson and Spielman, 1973; Raikhel and Lea, 1991). However, the present results suggest that 15a-2
contributes to the micropylar apparatus in this region. The formation of the *A. aegypti* micropylar apparatus has not been studied at the earliest stages. However, the site of 15a-2 expression is consistent with the formation of the micropylar apparatus in *Anopheles maculipennis*.

Nicholson (1921) found that the *A. maculipennis* micropylar apparatus was formed by a specialized group of follicle cells (rosette-cells), that are located in the anterior region of the follicle. During the period of vitelline membrane formation, globules that appear to be identical in appearance to vitelline membrane plaques, are secreted by the central rosette cells at the anterior pole. Vitelline membrane plaques are not secreted in the anterior region immediately surrounding the pole. The secretion of plaques at the pole continues until the formation of the vitelline membrane is complete. This isolated mass of vitelline membrane protein was called the "stopper" (of the micropyle) (Nicholson, 1921).

In *D. melanogaster*, an inner layer of the micropylar apparatus is formed by the border cells of the follicular epithelium. This layer is part of the vitelline membrane (Margaritis, 1985). Jin and Petri (1993) observed the expression of a reporter gene in the border cells of the follicular epithelium when a fragment of the upstream region of the vitelline membrane gene VM26A1 was introduced into *D. melanogaster* via P-element transformation.

**Role of ecdysone**

The formation of the *A. aegypti* vitelline membrane coincides with a sharp rise in 20-hydroxyecdysone (ecdysone) titer following the blood meal (Hagedorn et al., 1975; Gemmill et al., 1986). Raikhel and Lea (1982 and 1991) showed that the formation of the vitelline membrane (envelope) can be induced by the injection of ecdysone into decapitated females given blood by enema. Lin et al, (1993) demonstrated that vitelline membrane
gene expression was induced in the ovaries of the blood-fed, decapitated female in response to an injection of ecdysone.

The mechanism by which ecdysone regulates vitelline membrane gene expression is not understood. Is the continued exposure of ovaries to ecdysone necessary for vitelline membrane gene expression, or does the ecdysone initiate a regulatory program that continues independent of the hormone once the program has been established?

The effect of ecdysone on vitelline membrane gene expression was examined using an *in vitro* approach. Vitelline membrane gene expression was analyzed in ovaries that were dissected at increasing intervals following a blood meal and incubated *in vitro* without ecdysone for 10 hours. These data were compared to the levels expression in ovaries that were analyzed immediately following their dissection. The expression of vitelline membrane genes was followed, using a 15a-1 coding region probe that cross-reacts with other vitelline membrane genes. A composite northern blot of these experiments is shown in Fig. 4.5.

Vitelline membrane gene expression that was detected in ovaries after 10 h incubation without ecdysone was less than in ovaries that were analyzed immediately following their dissection at 10, 24 and 36 h PBM (Table 4.2). However, vitelline membrane genes were still expressed in the incubated groups at levels between 40 to 60 percent of the groups that were not incubated (Table 4.2). These data suggest that the continued presence of ecdysone is not necessary for the expression of vitelline membrane genes and/or these mRNA messages are stable for several hours. Further studies, such as the nuclear runoff assay, could assess the ratio of newly synthesized vitelline membrane mRNA to the total vitelline membrane signal that is detected in ovaries following 10 hours of incubation without ecdysone.
Effect of ecdysone in vitro

It has been previously shown that vitelline membrane gene expression was induced in ovaries that were dissected from non-blood fed mosquitoes and cultured for 24 hours in medium containing $10^{-5}$ M ecdysone (Lin et al., 1993). No vitelline membrane message was detected in ovaries that were cultured for 24 h in medium that did not contain ecdysone (Lin et al., 1993). These observations were confirmed with 10 h incubations (Tables 4.2 and Fig. 4.5). The signal that was detected in ovaries from non-blood fed mosquitoes incubated with ecdysone was about three orders of magnitude less than that of its maximal expression in vivo during the period of vitelline membrane formation (Table 4.2).

Levels of message were also compared in ovaries that were incubated in medium containing $10^{-5}$ M ecdysone and no hormone, respectively. In these experiments message levels declined in the absence of ecdysone, and rose when ecdysone was present in culture. However, by 36 h PBM, ecdysone no longer stimulated message levels. A similar pattern of responsiveness to ecdysone in vitro was observed with *A. aegypti* vitellogenin synthesis by Bohm et al., (1978). When fat bodies from non-blood fed females were incubated with $10^{-5}$ M ecdysone in vitro, vitellogenin synthesis reached a peak at 30 h and then declined even in the presence of hormone. Removal of hormone during the rising phase of vitellogenesis stopped vitellogenin synthesis. However, if ecdysone was replaced in the culture medium during the first 30 h of incubation, then vitellogenin synthesis resumed. In contrast, Ma et al., (1987) found that after 6 h of exposure to ecdysone, vitellogenin synthesis continued to rise independent of the presence of ecdysone.

Acknowledgments - This work was supported by NIH grant (HD24869) to Drs. Henry H. Hagedorn and Ann M. Fallon, and by the John D. and Catherine T. MacArthur Foundation.
TABLE 4.1. CPM values for time course Northern blot. The amount of radioactivity bound to individual samples on the nylon membrane was determined using a Betascope. Bound radioactivity was removed by several washes in 5% SDS at 100 °C. The effectiveness of the removal was determined using the Betascope. Residual CPM values that were detected after washing were subtracted from subsequent CPM values.

<table>
<thead>
<tr>
<th>Hours PBM</th>
<th>15a-1</th>
<th>15a-2</th>
<th>15a-3</th>
<th>18S RNA</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>201.6</td>
<td>4.3</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>3.1</td>
<td>233.4</td>
<td>22.0</td>
</tr>
<tr>
<td>7.5</td>
<td>0</td>
<td>0</td>
<td>3.1</td>
<td>169.1</td>
<td>27.6</td>
</tr>
<tr>
<td>10</td>
<td>1.6</td>
<td>8.3</td>
<td>9.3</td>
<td>158.5</td>
<td>17.1</td>
</tr>
<tr>
<td>15</td>
<td>5.4</td>
<td>23.2</td>
<td>60.2</td>
<td>216.6</td>
<td>34.6</td>
</tr>
<tr>
<td>20</td>
<td>19.2</td>
<td>50.1</td>
<td>91.3</td>
<td>145.3</td>
<td>28.7</td>
</tr>
<tr>
<td>25</td>
<td>72.0</td>
<td>140.0</td>
<td>236.2</td>
<td>123.2</td>
<td>20.2</td>
</tr>
<tr>
<td>30</td>
<td>148.5</td>
<td>230.9</td>
<td>481.8</td>
<td>156.1</td>
<td>29.4</td>
</tr>
<tr>
<td>35</td>
<td>177.9</td>
<td>269.3</td>
<td>502.2</td>
<td>144.1</td>
<td>16.6</td>
</tr>
<tr>
<td>40</td>
<td>233.1</td>
<td>298.3</td>
<td>477.8</td>
<td>155.3</td>
<td>28.8</td>
</tr>
<tr>
<td>45</td>
<td>167.0</td>
<td>207.2</td>
<td>230.0</td>
<td>136.4</td>
<td>28.1</td>
</tr>
<tr>
<td>50</td>
<td>23.7</td>
<td>47.3</td>
<td>15.2</td>
<td>141.5</td>
<td>25.2</td>
</tr>
<tr>
<td>55</td>
<td>1.7</td>
<td>6.4</td>
<td>1.9</td>
<td>154.5</td>
<td>32.0</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>90.5</td>
<td>28.2</td>
</tr>
</tbody>
</table>
TABLE 4.2. Effect of \textit{in vitro} incubation and ecdysone on 15-1 expression at increasing intervals PBM. Each experiment consists of three conditions: 1) No Incubation: Ovaries were dissected at the specified time PBM and total RNA was extracted. 2) Control Incubation: Ovaries were dissected at the specified time PBM and then cultured for 10 h in medium containing no hormone. 3) Ecdysone Incubation: Same as Control Incubation, but ovaries were incubated for 10 h in medium containing $10^{-5}$ M ecdysone. Approximately 12 $\mu$g of total RNA were loaded per lane of a Northern blot for experiments at 0, 2 and 10 h PBM, and 1.2 $\mu$g of total RNA was loaded per lane for experiments 24 and 36 h PBM. The northern blots were probed sequentially with 15a-l coding region probe and an 18S RNA probe. Values are reported as the mean CPM $\pm$ SE.

* Values are significantly different at P<0.05 (Student's t-test).
** Values are significantly different at P<0.01 (Student's t-test).
\(a\) Significantly different from "No Incubation" values at the same time interval.
\(b\) Significantly different from "Control Incubation" values at the same time interval.

<table>
<thead>
<tr>
<th>Hours PBM</th>
<th>No Incubation Mean CPM $\pm$ SE</th>
<th>Control Incubation Mean CPM $\pm$ SE</th>
<th>Ecdysone Incubation Mean CPM $\pm$ SE</th>
<th>18S RNA Mean CPM $\pm$ SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (n = 3)</td>
<td>0 (n = 3)</td>
<td>27.5 $\pm$ 4.0 (n = 3) <strong>b</strong></td>
<td>525.5 $\pm$ 26.1 (n = 9)</td>
</tr>
<tr>
<td>2</td>
<td>17.0 $\pm$ 3.8 (n = 3)</td>
<td>10.0 $\pm$ 0.5 (n = 4)</td>
<td>137.0 $\pm$ 20.5 (n = 6) <strong>b</strong></td>
<td>1143.4 $\pm$ 43.1 (n = 13)</td>
</tr>
<tr>
<td>10</td>
<td>261.9 $\pm$ 38.9 (n = 3)</td>
<td>59.3 $\pm$ 0.3 (n = 3) *a</td>
<td>556.9 $\pm$ 73.1 (n = 3) <strong>b</strong></td>
<td>455.7 $\pm$ 24.4 (n = 9)</td>
</tr>
<tr>
<td>24</td>
<td>599.2 $\pm$ 95.7 (n = 4)</td>
<td>239.5 (n = 2)</td>
<td>402.5 $\pm$ 16.5 (n = 3) *b</td>
<td>225.7 $\pm$ 12.7 (n = 9)</td>
</tr>
<tr>
<td>36</td>
<td>1564.1 $\pm$ 107.9 (n = 3)</td>
<td>955.0 $\pm$ 157.2 (n = 3) *a</td>
<td>772.8 $\pm$ 87.1 (n = 3)</td>
<td>198.4 $\pm$ 15.8 (n = 15.8)</td>
</tr>
</tbody>
</table>
FIGURE 4.1 Mutually exclusive probes for 15a-1, 15a-2 and 15a-3. Restriction fragments of cDNA (15a-1 and 15a-2) or genomic (15a-3) clones containing the respective 3'-untranslated regions were used in labeling reactions by extension from primers 1, 2 and 3 in the reverse (antisense) orientation.
FIGURE 4.2 Timing of vitelline membrane gene expression in ovaries following a blood meal. Total RNA was extracted from the ovaries at increasing intervals following a blood meal. Approximately 4 μg of total RNA were loaded per lane of a northern blot. The northern blot was probed sequentially with mutually exclusive 15a-1, 15a-2 and 15a-3, actin and 18S RNA probes.
FIGURE 4.3. Timing of 15a-1, 15a-2 and 15a-3 expression. The CPM values for 15a-1, 15a-2 and 15a-3 relative to the maximal values for expression at each time interval are profiled.
FIGURE 4.4, Site of 15a-1, 15a-2 and 15a-3 expression in ovaries at 24 h and 36 h PBM. Whole-mount *in situ* hybridization was performed using digoxigenin-labeled, mutually exclusive probes corresponding to the respective 3'-untranslated regions. a) Ovaries dissected at 24 h PBM and hybridized with 15a-2 probe (10X). b) Ovaries dissected at 24 h PBM and hybridized with 15a-1 probe (40X). c) Ovaries dissected at 24 h PBM and hybridized with 15a-2 probe (40X). d) Ovaries dissected at 36 h PBM and hybridized with 15a-3 probe (40X). e) Ovaries dissected at 36 h PBM and hybridized with 15a-1 probe (100X). Arrow shows weak hybridization with 15a-1 probe in cell at anterior pole. f) Ovaries dissected at 36 h PBM and hybridized with 15a-2 probe (100X).
FIGURE 4.5 Effect of *in vitro* culture and ecdysone on vitelline membrane gene expression at increasing intervals PBM. Each experiment shown in this composite figure of several northern blots consists of three conditions. 1) No Incubation (NI): Ovaries were dissected at the specified time PBM and total RNA was extracted. 2) Control Incubation (CI): Ovaries were dissected at the specified time PBM and then incubated for 10 h in medium containing no hormone, prior to RNA extraction. 3) Ecdysone incubation (EI): Same as CI, but ovaries were incubated for 10 h in medium containing $10^{-5}$ M 20-hydroxyecdysone. Approximately 12 µg of total RNA were loaded per lane of northern blot at 0, 2 and 10 h PBM and 1.2 µg of total RNA was loaded per lane at 24 and 36 h PBM. The northern blots were probed sequentially with a 15a-1 coding region probe and an 18S RNA probe.
Plasmids were constructed that contain sequences upstream of three *Aedes aegypti* vitelline membrane genes, 15a-1, 15a-2 and 15a-3 and a reporter gene, β-galactosidase. Future investigations will determine whether 20-hydroxyecdysone induces the expression of β-galactosidase in cultured *Aedes albopictus* C7-10 cells that have been transfected with the 15a-1, 15a-2 and 15a-3 constructs.
INTRODUCTION

DNA mediated gene transfer is one approach to study the transcriptional regulation of genes. Germ line transformation via the transposable element P (P-element) has been used to study the regulation of three *Drosophila melanogaster* vitelline membrane genes. In separate studies, regulatory DNA sequences upstream of VM26A1, VM26A2 and VM32E were linked to a lacZ reporter gene, and the constructs were introduced into *D. melanogaster* by P-element transformation (Savant and Waring, 1989; Gargiulo et al, 1993; Jin and Petri, 1993).

Transforming *A. aegypti* with constructs containing the vitelline membrane upstream regions and reporter genes is one approach to study the regulation of these genes. However, reliable transformation methods have not been developed for use with *A. aegypti*, as they have with *D. melanogaster*. Alternatively, the upstream regions of the *Aedes* vitelline membrane genes could be introduced into *Drosophila* via P-element transformation. However, this approach is labor intensive and the significance of both positive and negative results are difficult to interpret.

Another approach to study the regulation of the *A. aegypti* vitelline membrane genes is to transfect an *A. aegypti* cell line with reporter constructs. Vitelline membrane formation is regulated by 20-hydroxyecdysone (Raikhel and Lea, 1982 and 1991). Vitelline membrane gene expression can be induced by ecdysone in ovaries at some stages of development *in vitro* (Lin et al, 1993). Thus the effect of ecdysone on vitelline membrane expression will be examined in an *Albopictus albimanus* cell line C7-10. Ecdysone induces the expression of at least three proteins in these cells (Dr. Ann Fallon. *personal communication*).
METHODS

Vectors

The 15a-1, 2 and 3 reporter constructs were based on plasmid pKS+BZT, which was previously constructed by Peter Ke (University of Arizona) (Figure 5.1). The construct pKS+BZT contained a promoterless lacZ gene encoding β-galactosidase, which was followed by a poly-A terminator signal of the hsp 70 gene. The pKS+BZT construct also contained 2.4 kb restriction fragment (B-fragment) containing sequences upstream of the Aedes vitellogenin gene VgA1. The B-fragment also contained several nucleotides within the open reading frame of VgA1. The B fragment was subcloned into the construct so that it was in the correct 5'- to 3'- orientation, and so that the initiation codon of the B-fragment was in the same reading frame as the lacZ structural gene. This placement allowed for the transcription of the E. coli β-galactosidase gene from the A. aegypti VgA1 promoter. The transcription of β-galactosidase can be detected using the chromogenic substrate, X-gal.

Preparation of the constructs

The strategy used to prepare the 15a-1, 15a-2 and 15a-3 constructs is shown in Figure 5.2. Restriction enzyme sites were not situated within the these sequences that would permit their ligation with the lacZ gene. Therefore, restriction sites were introduced into the sequences by adding restriction sites into primers that were subsequently used in the polymerase chain reaction (PCR). Forward primers were designed upstream of the coding regions. Reverse primers were designed to correspond to nucleotides encoding amino acids within the coding region of the 15a-1, 15a-2 and 15a-3 genes. The reverse primers all included an in-frame Pst I site. The forward primers included Xho I sites.
The PCR was performed under standard conditions with 15 cycles in order to minimize errors in incorporation. The PCR products were subcloned into the pCR™II cloning vector (Invitrogen), which allowed for their excision from its multiple cloning site with the restriction enzymes Pst I and Xho I. This restriction fragment was ligated to a 6.2 kb Pst I-Xho I restriction fragment of the pKS+BZT plasmid. The 6.2 kb fragment contained pBluescript KS- vector, the promoterless lac Z gene, the hsp70 terminator sequence, but not the B fragment of VgA1.

The resulting constructs were used to transform a strain of *E. coli* (One Shot™, Invitrogen). Transformants containing plasmids with the vitelline membrane gene promoter in-frame with the lacZ gene resulted in blue colonies on agar plates containing X-gal, due to the expression of the lacZ gene. The DNA sequences contained within selected plasmids were verified by restriction analysis and PCR. They were also verified by sequencing in the 3' direction from the coding region of the vitelline membrane gene, through the pCR™II and pBluescript polylinker sites and into the lacZ sequence.

**Preparation of the 15a-1 construct**

The template for the 15a-1 reaction was a 2.1 Hind III fragment of the 15a-1 genomic clone containing upstream sequences. The forward primer was designed 1.8 kb upstream of the translational initiation site. The reverse primer was designed to contain the sequence encoding amino acids 7-14 as well as an in-frame Pst I site which was not present in the 15a-1 sequence. The 1.8 kb PCR product was subcloned into the pCR™II vector. A pCR™II subclone with an insert in the correct orientation with respect to its multiple cloning site was selected by restriction analysis. The insert from this plasmid was isolated from the pCR™II vector by partial digestion with the Pst I and Xho I.
This construct was used to transform a strain of *E. coli* (One Shot ™, Invitrogen). Transformants containing constructs with the 15a-1 promoter region that was in-frame with the lacZ gene were selected as blue colonies on agar plates containing X-gal. The constructs were verified by restriction analysis and PCR using previously designed primers. They were also verified by sequencing in the 3' direction from the coding region of 15a-1, through the pCR™II and pBluescript polylinker sites and into the lacZ sequence. The construct contained no mismatches, and the lacZ sequence was shown to be in frame (Figure 5.3).

**Preparation of the 15a-2 construct.**

The preparation of the 15a-2 construct was similar to that of 15a-1, thus only the differences will be described in this section. The template for the 15a-2 PCR was a 3.7 Kb Eco RI restriction fragment of genomic clone containing 15a-2 upstream sequences. The forward primer was designed 2.2 kb upstream of the translational initiation site. This primer contained an additional Xho I site that was not contained within the 15a-2 upstream sequence. The reverse primer corresponded to the sequence encoding amino acids 6-13, and contained an in-frame Pst I site. This 2.2 kb PCR product was also subcloned into pCRII, and the insert was isolated by complete digestion of the plasmid with Pst I and Xho I. This fragment was ligated to a 6.2 kb Pst I-Xho I fragment of pKS+BZT containing the lacZ gene and hsp terminator sequences. The construct was verified by restriction analysis, PCR and sequencing in 5' to 3' direction through the 15a-2 sequence and pBluescript polylinker into the lacZ gene. The sequence of the 15a-2 construct contained no mismatches, and lacZ sequence was in frame (Figure 5.4).
Preparation of the 15a-3 construct

The preparation of the 15a-3 construct was similar to that of 15a-1, thus only the differences will be described in this section. The template for the 15a-3 PCR was the 15a-3 genomic clone, that was purified according to Sambrook et al., (1989). The forward primer was designed 2.0 kb upstream of the translational initiation site. The reverse primer corresponded to the sequence encoding amino acids 7-14, and contained an in-frame Pst I site. The resulting 2.0 kb fragment was subcloned into pCRII (Invitrogen). A 2.0 kb Pst I- Xho I site was obtained by partial digestion of the recombinant plasmid. This was ligated with a 6.2 kb fragment of pKS+BZT containing the lacZ gene and hsp terminator sequences. The construct was verified by the formation of blue colonies on X-gal agar plates, restriction analysis, PCR and sequencing in the 5' to 3' direction through the 15a-3 sequence and pBluescript polylinker into the lacZ gene. The sequence of the 15a-3 construct contained no mismatches, and lacZ sequence was in frame (Figure 5.5).
pKS+BZT Construct

FIGURE 5.1. Reporter construct containing DNA sequence upstream of the A. aegypti vitellogenin gene VgA1, and ligated in frame with a β-galactosidase gene. This construct contains a 70 bp intron, which places the β-galactosidase gene out of frame with the translational start site. pKS + BZT was originally constructed by Dr. Peter Ke (University of Arizona).
1. Generate PCR product with Pst I site contained at 5’ end of reverse primer

2. Ligate with pCR II vector (3932 nucleotides, Invitrogen).

3. Determine which subclone has insert in the correct orientation.

4. Incomplete digestion of the correctly oriented TA subclone with Pst I + Xho I will provide a fragment that can be ligated to the pHISPZT 6.2 Kb Pst I + Xho I fragment.

5. Ligate with pKS +BZT Xho I + Pst I 6.2 Kb fragment

6. Analyze completed construct

FIGURE 5.2 Cloning strategy for reporter constructs with DNA sequences upstream of A. aegypti VgAI vitellogenin, and vitelline membrane genes 15a-1, 15a-2 and 15a-3.
15a-1 Construct

![Diagram of DNA construct]

FIGURE 5.3. Reporter construct containing DNA sequence upstream of vitelline membrane gene 15a-1, and ligated in frame with the lac Z gene encoding β-galactosidase. Sequence upstream of 15a-1 was obtained by PCR, using primers P. 101 and P. 100, using restriction fragments containing the 15a-1 upstream region as template.
15a-2 Construct

FIGURE 5.4. Reporter construct containing DNA sequence upstream of vitelline membrane gene 15a-1, and ligated in frame with the lac Z gene encoding β-galactosidase. Sequence upstream of 15a-2 was obtained by PCR, using primers P. 104 and P.103, using restriction fragments containing the 15a-2 upstream region as template.
**15a-3 Construct**

FIGURE 5.5 Reporter construct containing DNA sequence upstream of vitelline membrane gene 15a-3, and ligated in frame with the lac Z gene encoding β-galactosidase. Sequence upstream of 15a-3 was obtained by PCR, using primers P. 13 and P.105 and 15a-3 genomic clone phage DNA containing the 15a-3 region as template.
CHAPTER VI

MAPPING THREE VITELLINE MEMBRANE GENES ONTO THE Aedes aegypti CHROMOSOMES

INTRODUCTION

The relative timing of expression of the A. aegypti vitelline membrane genes 15a-1, 15a-2 and 15a-3 is very similar (Chapter III). One explanation for the coordinate temporal expression is that the three genes may be located within a single locus on the chromosome. Thus, a single regulatory mechanism affecting chromatin structure could be involved in the expression of all three of the genes. In order to test this hypothesis, these genes are being mapped onto the A. aegypti chromosomes in a collaborative effort with Dr. David Severson at the University of Wisconsin (Madison, WI).

The construction of an A. aegypti genetic linkage map based on restriction fragment length polymorphisms (RFLPs) was described by Severson et al., (1993). RFLP analysis employs cloned sequences to probe specific regions of the genome for variations at the DNA level. Polymorphisms are defined as differences in the length of DNA fragments observed following digestion with restriction endonucleases.

The A. aegypti RFLP linkage map consists of more than 50 DNA markers that identify more than 53 loci covering 134 map units across three linkage groups. The determination of linkage associations between RFLP markers and several mutant marker loci allowed for a partial integration of the RFLP markers with an existing classical genetic linkage map for A. aegypti. The RFLP markers include 42 random cDNA clones, three random genomic DNA clones and five cDNA clones of known genes (Severson et al. 1993).
METHODS

*Mutually exclusive probes*

Antisense (reverse) and sense (forward) primers were designed based on the predicted 3'-region of the 15a-1, 2 and 3 transcriptional units. These regions were amplified by the polymerase chain reaction (PCR) and the PCR products (200, 160 and 230 nucleotides in length respectively) were subcloned into the vector pCR™II (Invitrogen). PCR reactions were carried out in 1X PCR buffer, 1.6 mM MgCl₂, 0.1 mM dNTPs 0.5μM of each primer and 1.25 units of Taq polymerase. Taq polymerase was added at 75 °C. Eco RI fragments of the subcloned PCR products corresponded to the 3'-untranslated region and used for hybridization probes.

RESULTS

An *A. aegypti* cross has been performed in which 15a-1 and 15a-2 clones show polymorphisms. Preliminary map data has been obtained for the 15a-1 clone. It seems to be near the end of chromosome 3, opposite the locus for black tarsus. Data for other loci in this linkage group are presently being generated.
SUMMARY

The process of egg development in A. aegypti involves a complex program of gene expression. In this context, three genes encoding vitelline membrane (envelope) proteins were analyzed. Cloning these genes has allowed for a comparative analysis of their deduced peptide sequences as well as the construction of molecular probes to study their regulatory control.

A comparison of the three deduced vitelline membrane peptide sequences revealed regions of 45 residues within each sequence that are highly conserved. The conserved region overlaps with a peptide sequence that is conserved between four Drosophila melanogaster vitelline membrane genes. The overlapping region contains three conserved cysteine residues. It is possible that the conserved regions provide a structural conformation that is favorable to the formation of intermolecular disulfide bridges.

Are the vitelline membrane genes coordinately expressed? If 15a-1, 15a-2 and 15a-3 are expressed with the same spatial and temporal patterns following a blood meal, then it is possible that they share a common regulatory control mechanism. Such a mechanism could potentially be revealed by a comparative analysis of the three genes.

The relative timing of 15a-1, 15a-2 and 15a-3 expression was determined by Northern analysis using mutually exclusive probes. The respective expression profiles were similar although apparently not identical. The relative sites of 15a-1, 15a-2 and 15a-3 expression were determined by whole-mount in situ hybridization, using digoxigenin-
labeled mutually exclusive probes. Unlike the timing of expression, differences were detected between the respective sites of expression. Expression of 15a-1 and 15a-3 was strong in the mid- and posterior regions of the follicular epithelium but either weak or not detectable in the anterior region. In contrast, 15a-2 expression was strongest in the anterior region of the follicle. It is possible that 15a-2 may contribute to the formation of the micropyle. The differences in spatial expression between the three vitelline membrane genes suggests that they do not share a common mechanism of regulatory control.

The formation of the vitelline membrane coincides with a rise in 20-hydroxyecdysone (ecdysone) titre that occurs following a blood meal. What is the role of ecdysone in the regulation of vitelline membrane gene expression? This question was analyzed by Northern analysis of ovaries that were dissected from females at increasing intervals following a blood meal. The ovaries were incubated for 10 h in culture medium containing either 10^{-5} M ecdysone or no hormone. As compared to control incubations, ecdysone stimulated the expression of vitelline membrane genes from 0 to 24 h after a blood meal. Ecdysone had no effect on vitelline membrane gene expression in ovaries that were dissected at 36 h after the blood meal. It is possible that ecdysone does not stimulate vitelline membrane gene expression after the hormone reaches its peak titre at 24 h after a blood meal.

DNA sequences upstream of the three vitelline membrane coding regions were analyzed for potential regulatory elements. Sequence similarity was detected within the promoter regions of the three genes. No similarity was detected upstream of the three promoter regions. Since ecdysone was shown to have a role in the regulation of vitelline membrane gene expression, sequences with similarity to an ecdysone responsive element consensus sequence were identified upstream of the 15a-1, 15a-2 and 15a-3 coding regions. One of the consensus sequences was present within the 360 bp region that was
72% similar between the 15a-2 and *A. aegypti* VgA1 vitellogenin gene upstream sequences.

This study has enhanced the resolution of what is known about the *A. aegypti* vitelline membrane to the molecular level. Molecular probes have indicated regional expression differences within the follicular epithelium that could not be detected by ultrastructural studies. Progress has been made towards an understanding of the regulation of vitelline membrane gene expression.
How is the expression of the *A. aegypti* vitelline membrane genes regulated? The tissue- and stage- specific expression patterns of the *A. aegypti* vitelline membrane genes are regulated by the interactions of proteins with cis-regulatory DNA sequences. However, in this system, neither the regulatory DNA sequences nor the trans- acting proteins are known. A comparative analysis of the 15a-1, 15-2 and 15a-3 upstream sequences has not revealed any candidates for regulatory motifs on the basis of consensus between the sequences. However, three methods could be used to identify regulatory proteins and DNA motifs when neither are known a priori.

In the gel mobility shift assay, interactions between upstream DNA fragments and regulatory proteins could be detected. *In vitro* methylation interference assays could be done with the DNA fragments which are identified in the gel mobility shift assay. Residues implicated in interactions with regulatory proteins could be confirmed by DNase I footprinting.

**Mobility shift assays**

One of the most widely applied methods for the detection of protein-DNA interactions has been the mobility shift assay (Chodosh, 1988). The premise of the gel shift assay is that proteins that bind to a labeled fragment DNA will retard its PAGE mobility, when compared to free DNA. The gel mobility shift has been used in several studies that are investigated the regulation of ecdysone responsive genes. Koelle et al, (1991) first isolated the ecdysone receptor using the gel mobility shift as a primary tool. Thomas et al, (1993) showed that the active EcR is formed from a heterodimer of the protein, ultraspiracle and an EcR monomer using the gel mobility shift assay. The assay
has also been used to detect chorion factors 1 and 2 (CF1 and CF2) that bind to D. melanogaster s15 chorion cis-regulatory elements (Shea et al, 1990).

**In vitro methylation interference assay**

The *in vitro* methylation assay adds another layer of precision to the gel mobility shift assay (Baldwin, 1988). It can be used to identify individual guanine residues that are involved in the binding of *trans*-acting factors. For this assay, fragments of upstream DNA that have been shown to be effective with the gel shift assay are end-labeled. The labeled fragments of DNA are then exposed to the methylating agent, dimethylsulfate (DMS). This compound methylates guanine residues. Protein extracts which have been used in the gel shift assay are then added to the methylated DNA. Following this, a gel shift assay is performed with the DMS treated, end-labeled DNA and protein mixtures. Shifted and non-shifted bands are then cut out of the gel, and the DNA is eluted from the polyacrylamide matrix. The DNA is then treated with piperidine, which cleaves the DNA at methylated guanine residues. Subsequently, the piperidine-cleaved DNA samples are electrophoresed on a sequencing gel.

The results of the methylation interference experiment are interpreted by comparing the guanine residues between the shifted and non-shifted fragment samples as they appear on a sequencing gel. Guanine residues in the shifted band were not methylated because they were protected by a protein interaction, thus the DNA is not cleaved by piperidine at these residues. Guanine residues that are protected from methylation by regulatory proteins do not appear on the sequencing gel.
DNase I footprinting

DNase I footprinting is a method used to analyze interactions of proteins that are tightly associated with specific DNA sequences. Like the in vitro methylation assay, it is a refinement of the gel mobility shift assay. In this assay, the binding of proteins to DNA protects it from a cleavage by DNase I, instead of the combined actions of DMS and piperidine (Brenowitz et al., 1988). The results of this experiment are interpreted by the lack of guanine and adenine residues in the ladder of DNase I-nicked DNA. These gaps correspond to the phosphodiester bonds that were protected from single-stranded DNase I-mediated DNA nicks by the binding of associated proteins.

The DNase I footprinting assay was one of the primary methods used by Riddihough and Pelham (1991), who were the first to report an ecdysone responsive element 100 bp upstream of the D. melanogaster hsp27 gene. Georgel et al., (1991) used the technique of DNase I footprinting to study the binding of a trans-acting factor, glue enhancer binding factor 1 (GEBF-1), and its relation to the ecdysone induction of a glue gene enhancer sequence. Similarly, DNase I footprinting was used to determine the properties of doublesex protein binding to the ovarian and fat body enhancer sequences (Burtis et al, 1991).
REFERENCES


