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Isolation and chromosomal location of putative vitelline membrane genes in *Drosophila melanogaster*

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cDNA clones for two *Drosophila* vitelline membrane genes have been identified on the basis of: (i) stage and tissue specificity of transcription and (ii) size and amino acid content of the translation product. Cross-hybridization data suggest that DmcMM99 and DmcMM115 are members of a multi-gene family which includes at least three members, all of which reside on the left arm of the second chromosome. DmcMM99 and DmcMM115 originate from polytene band positions 34C and 26A, respectively. A third, cross-hybridizing gene resides at position 32EF. Southern analysis of a genomic clone, λLS1, homologous to DmcMM115, indicates that two vitelline membrane genes may be clustered at the 26A site.
Key words: base sequence/*Drosophila* clones/*in situ* hybridization/multi-gene family/vitelline membrane genes

Introduction

We are particularly interested in studying the structure, organization and expression of the genes responsible for the proteins of the first layer of the *Drosophila* eggshell, the vitelline membrane (VM). Both cytological (e.g., Quattropani and Anderson, 1969; King, 1970; Margaritis *et al.*, 1980) and biochemical data (Petri *et al.*, 1976; Fargnoli and Waring, 1982) indicate that the VM proteins are synthesized specifically during the vitellogenic stages of follicle development; i.e., stages 8, 9 and 10. Data from Spradling and Mahowald (1979) suggest that VM mRNA shows a parallel stage specificity. Hence, we expect transcription and translation of VM genes to be closely coupled. Concomitant accumulation of mRNA and protein has been documented for the second layer of the eggshell, the chorion (Spradling and Mahowald, 1979; Griffin-Shea *et al.*, 1982).

Recently we constructed a cDNA library in plasmid pUC9 from poly(A)$^+$ RNA extracted from *Drosophila melanogaster* egg chambers in all stages of oogenesis. Results of a developmentally differential screening of this library have shown it to to be rich in sequences which are expressed in a stage-specific manner during oogenesis (Mindrinos, 1984; Mindrinos, Jacobs and Petri, in preparation). We found one class of these sequences whose expression is primarily limited to the prechoriogenic stages (prior to stage 11) when the VM proteins are synthesized. DmcMM99 and DmcMM115 are two plasmid cDNA clones in this prechoriogenic class: both contain sequences that are represented in cDNA made from stage 1–10 follicular RNA, but not in reverse transcribed RNA from stages 11–14. Previous studies (Mindrinos, 1984) have demonstrated that sequences carried by both clones: (i) show adult tissue-specific expression limited to the ovary and (ii) are not represented in the maternal RNA of the mature oocyte or early embryo. In addition, neither DmcMM99 nor DmcMM115 reveals homology to probes for *Drosophila* yolk proteins (Barnett *et al.*, 1980) or tubulin-IV (Kalfayan and Wensink, 1982). Here we report in detail on characteristics of DmcMM99 and DmcMM115 which indicate that these clones represent two *Drosophila* VM genes.

Results

Size and homology of transcripts encoded by DmcMM99 and DmcMM115

The size of mRNAs complementary to DmcMM99 and DmcMM115 was determined by Northern blot analysis of total ovarian RNA (Figure 1). When relatively stringent hybridization critera were employed (55°C), a single transcript of ~650 bp was observed for DmcMM99 and a single transcript of ~750 bp was detected for DmcMM115. When more permissive hybridization conditions were used (37°C), DmcMM99 probes hybridized with two additional transcripts (Figure 1a, open triangles). Two additional transcripts were also detectable using a DmcMM115 probe under the more permissive conditions; however, the low signal-to-background ratio in the case of the higher mol. wt. cross-hybridizing species makes photographic presentation difficult. Since the lower mol. wt. cross-hybridizing species for DmcMM99 and DmcMM115 are of the same transcript size as the major transcript of the opposite clone and since both clones appear to have a common high mol. wt. cross-hybridizing transcript, some degree of homology between these two clones is suggested. This conclusion is supported by the observation that the DmcMM115 insert cross-hybridizes under permissive hybridization criteria with the DmcMM99 plasmid (data not shown).

Stage-specific expression of transcripts

If DmcMM99 and DmcMM115 represent VM sequences, we predict that their transcription would be limited primarily to stages 8, 9 and 10. A developmental Northern analysis of DmcMM99 and DmcMM115 (Figure 2) reveals the expected pattern of expression. The same nitrocellulose filter was hybridized sequentially first with a DmcMM99 (Figure 2a) and afterwards with a DmcMM115 probe (Figure 2b). Both hybridization reactions were performed under permissive conditions in an attempt to identify the developmental expression pattern for all cross-hybridizing species as well as for the major transcript in each case. The results show that the major 650-bp and 750-bp transcripts encoded by DmcMM99 and DmcMM115, respectively, are present specifically in stages 8, 9 and 10. In addition, the high mol. wt. cross-hybridizing transcript is also most abundant in stage 10; longer X-ray film exposures indicate that it too is present at lower levels in the earlier vitellogenic stages.

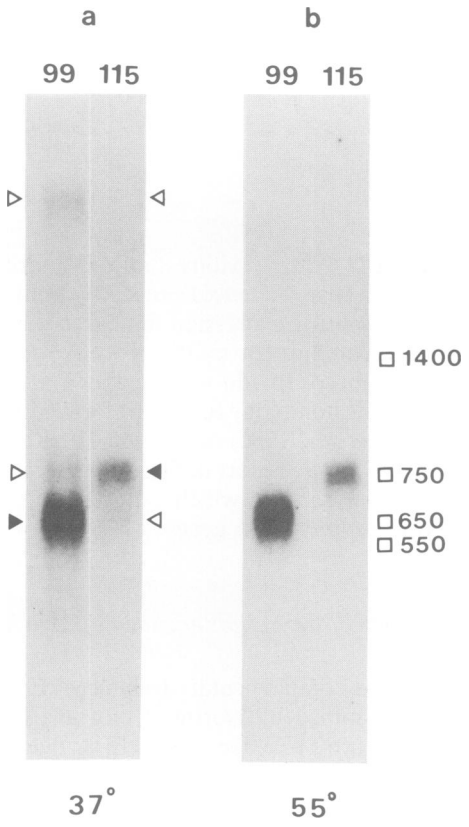

Fig. 1. Identification of transcript sizes encoded by DmcMM99 and DmcMM115. 10 μg of RNA from ovaries enriched in stages 1 – 10 were electrophoresed in a formaldehyde-containing gel, blotted onto nitrocellulose paper and parallel lanes hybridized in 50% formamide, 4 x SSC to nick-translated plasmids (99 = DmcMM99; 115 = DmcMM115) at two different temperatures, **(a)** 37°C and **(b)** 55°C. Squares indicate the parallel migration positions of mRNA encoding the s16, s19 and s38 chorion proteins with known sizes of 650, 750 and 1400 bp, respectively. Encoded transcripts are indicated by solid triangles and cross-hybridizing species by open triangles.

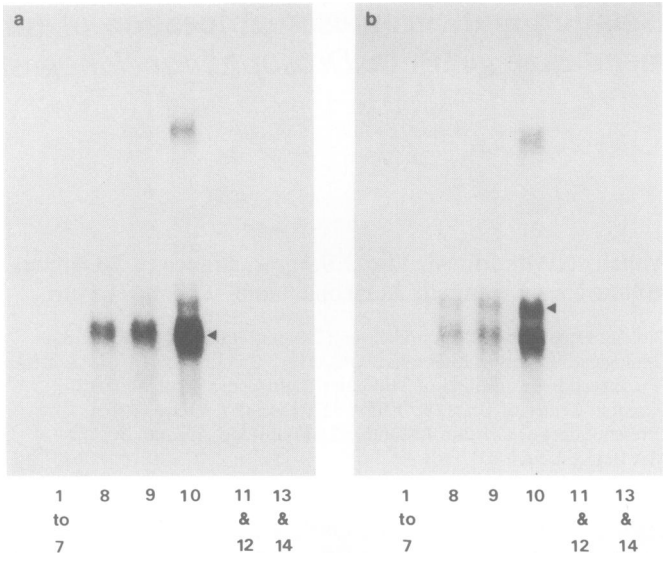

Fig. 2. Developmental accumulation of RNAs corresponding to DmcMM99 and DmcMM115. Total RNA from 400 – 500 stage 1 – 7 egg chambers, 260 stage 8, 130 stage 9, 65 stage 10, 65 stage 11 – 12 and 65 stages 13 – 14 were electrophoresed in a formaldehyde gel and blotted onto nitrocellulose paper. The paper was sequentially hybridized with DmcMM99 **(a)** and then DmcMM115 **(b)** without interim stripping of the filter with nick-translated plasmids at 37°C in 50% formamide, 4 x SSC. Solid triangles indicate the position of the 650 bp **(a)** and 759 **(b)** transcripts encoded by DmcMM99 and DmcMM115, respectively. Egg chamber stages are indicated below each gel lane.

VM proteins and cell-free translation patterns from ovarian RNA

To show that DmcMM99 and DmcMM115 encode VM proteins, it is important to identify and characterize these proteins and their precursors. A number of VM proteins have already been identified based, in part, on their expected stage-specific pattern of synthesis (Petri *et al.*, 1976; Fargnoli and Waring, 1982; Mindrinos, 1984). Figure 3a shows a partial developmental series from whole egg chambers labelled in culture with [^{3}H]proline. Relevant components which have been previously identified as VM are labelled (v10, v11, v13 and v14.5 and v100) according to their approximate kilodalton mol. wts. Proteins extracted from dissected and carefully hand-purified VM from ^{3}H-labelled ovaries are seen in Figure 3b. The background of components non-specific to stages 8 – 10 seen in Figure 3a has disappeared, revealing many of the VM proteins. The relatively low levels of v10 and v21 seen in Figure 3b is, in part, a consequence of the particular stage distribution of egg chambers present in the ovaries at the time of radiolabelling. v13, in particular, is relatively under-represented in this mixed stage preparation because, unlike other VM components, its biosynthesis is limited to stage 10B (Mindrinos, 1984; Mindrinos and Petri, in preparation).

We find that the three major translation products (pVi, pVii and pVIii) of mRNA derived from stage 8 – 10 egg chambers and translated in a wheat germ system migrate at positions ~1000 – 2000 daltons larger than major, mature VM proteins of low mol. wt. (v11, v12, v14.5; Figure 3c). These three proteins are absent in translations of total RNA from stages 11 – 14 as would be expected for VM components (data not shown). The observed size discrepancy can be explained if the VM proteins, like the chorion proteins, have a signal peptide removed during secretion (Blobel and Dobberstein, 1975a, 1975b). Thireos *et al.* (1979) and Spradling *et al.* (1980) have demonstrated pre-secretory chorion precursor proteins for all chorion components tested. In all cases, chorion protein precursors were 1000 – 3000 daltons larger than their mature counterparts.

Hybrid select translations

To determine if DmcMM99 and DmcMM115 encode proteins which correspond to VM proteins, each clone was used to hybrid-select total ovarian RNA (Figure 4). The cell-free translation product of the selected RNA indicates that DmcMM99 encodes a protein which corresponds to pVi while DmcMM115 corresponds to pViii. This result is consistent with both clones encoding precursors for VM components.

Base sequencing and translation product of DmcMM99

Further confirmation that DmcMM99 represents a VM protein was obtained from base sequencing data. In general, the VM proteins contain a distinguishing profile of amino acids (Petri *et al.*, 1976). Most notably they are rich in proline and alanine. Hence, if these clones do indeed represent VM genes, we expect them to encode proteins whose amino acid composition reflects these data. To confirm this, the DNA sequence of DmcMM99 was obtained (Figure 5). We find that the only open reading frame does indeed code for a protein which would be relatively high in proline and alanine and which would have an overall amino acid content consis-

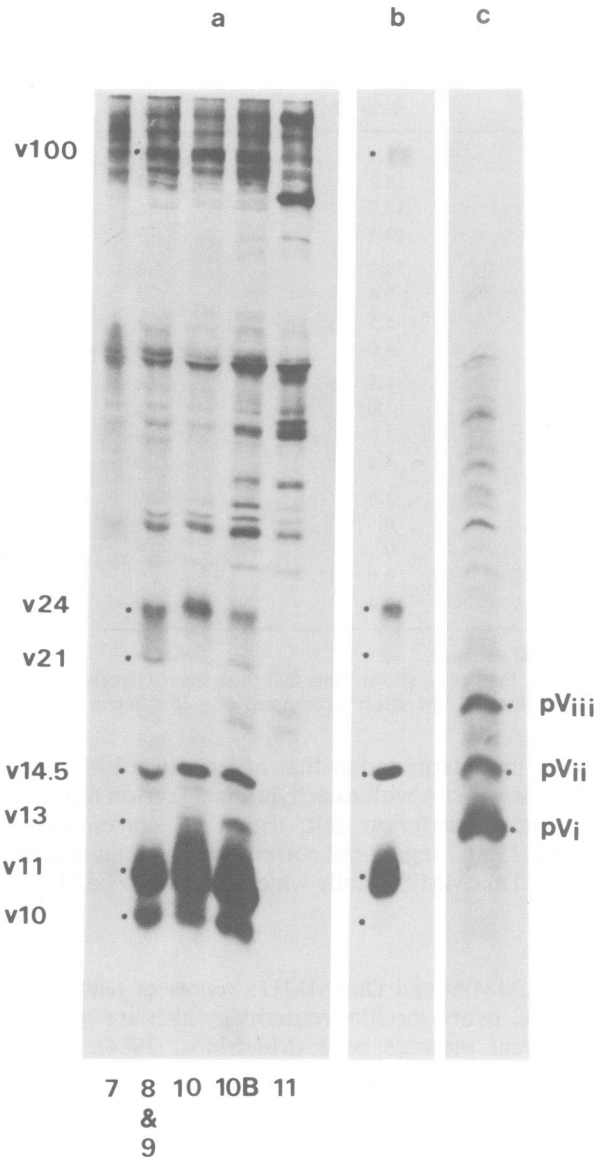

Fig. 3. Identification of precursor proteins for VM components. Proteins were labelled with [^{3}H]proline, dissolved in Laemmli buffer and analyzed on 15% acrylamide gels. **(a)** Egg chamber stages 7, 8–9, 10 (combined 10A and 10B), 10B and 11 labelled in separate groups in Robb's culture media (Robb, 1969) for 1 h and run in the lanes indicated at the bottom of the figure. **(b)** VM purified from ovaries labelled in Robb's culture media for 1 h. **(c)** Protein profile from stage 8–10 egg chamber RNA translated in a wheat germ system. Previously identified VM components are labelled with a 'v' prefix and putative VM precursor proteins are labelled with 'pV'.

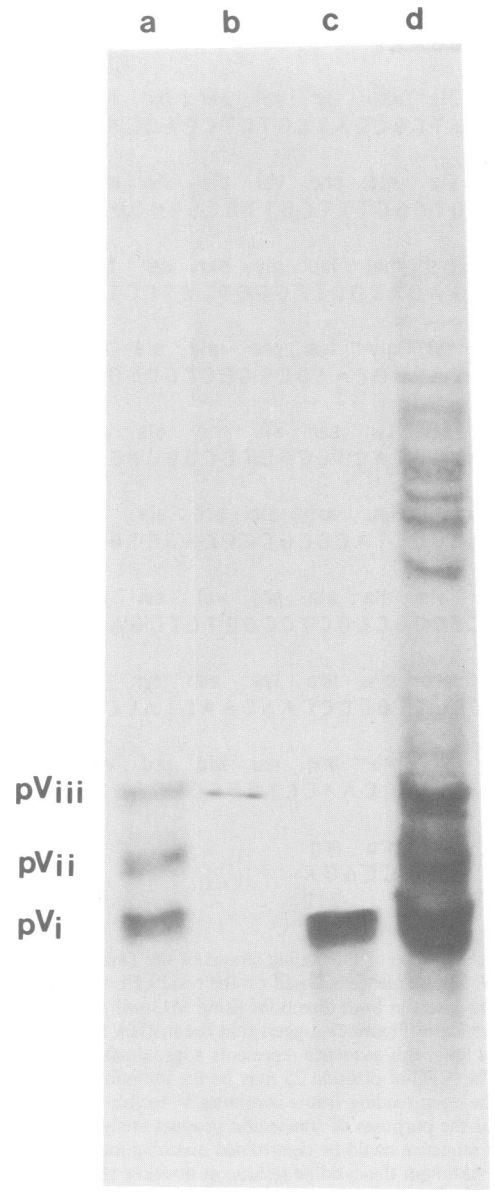

Fig. 4. Identification of proteins encoded by DmcMM99 and DmcMM115. **(a)** Protein profile of RNA from egg chamber stages 8–10 translated in a wheat germ system similar to Figure 3a. **(b)** Translation product of RNA hybrid-selected by DmcMM115. **(c)** Translation product of RNA hybrid-selected by DmcMM99. **(d)** Translation products of total RNA from ovarian tissues used for hybrid selection by DmcMM99 and DmcMM115. Putative VM precursor proteins are denoted with the prefix 'pV'.

tent with the average found for the total VM which is composed of ~10 different proteins (Table I). Given the homology between DmcMM99 and DmcMM115, this result strongly implies that both clones contain VM sequences.

In situ hybridization of DmcMM99 and DmcMM115

Chromosomal locations for our putative VM genes were determined by hybridizing nick-translated DmcMM99 and DmcMM115 probes *in situ* to salivary gland chromosomes (Figure 6). In each case one major site of hybridization was observed as well as two relatively minor sites (Table II), all on chromosome 2L. Given the data from Figures 1 and 2 in conjunction with this result, we conclude that the gene corresponding to DmcMM99 resides at polytene band location 34C and the gene corresponding to DmcMM115 at 26A. In addition, the data are consistent with the possibility that the gene corresponding to the cross-hybridizing, high mol. wt. transcript resides at 32EF.

Selection and mapping of a genomic clone for DmcMM115

The six major chorion genes are known to be divided into two chromosomal clusters at 7F1-2 on the first chromosome and at 66D10-15 on the third (e.g., see Spradling, 1981; Yannoni and Petri, 1981; Griffin-Shea *et al.*, 1982). In order to investigate the possibility that multiple VM genes likewise are clustered at some of the VM chromosomal locations which we had established, we used DmcMM115 to select an ~14-kb genomic clone, λLS1, from a lambda *Drosophila* library (Figure 7). Southern analysis of λLS1 using DmcMM115 as a

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 ser  gly  asn  his  gln  pro  ile  asn  met  lys
TCTGGAAATCATCAACCAATCAACATGAAG
1                                              30
 cys  ile  ala  ile  val  ser  thr  leu  cys  leu
TGCATCGCCATCGTCTCCACCATCTGCCTG
31                                             60
 leu  ala  ala  phe  val  ala  ala  asp  lys  glu
CTGGCCGCTTTCGTTGCCGCCGATAAGGAG
61                                             90
 asp  lys  met  leu  gly  ser  ser  tyr  gly  gly
GATAAGATGCTCGGCTCCTCCTACGGTGGT
91                                            120
 gly  tyr  gly  lys  pro  ala  ala  ala  pro  ala
GGCTACGGCAAGCCCGCCGCTGCTCCGGCT
121                                           150
 pro  ser  tyr  ser  ala  pro  ala  ala  ala  ser
CCATCCTACTCCGCTCCGGCTGCCGCTTCC
151                                           180
 pro  gly  leu  arg  ala  pro  ala  ala  pro  ser
CCAGGCCTACGCGCCCCAGCTGCTCCATCC
181                                           210
 tyr  ala  ala  ala  pro  val  ser  ile  pro  ala
TACGCCGCCGCTCCGGTCTCGATCCCGGCT
211                                           240
 pro  pro  cys  pro  lys  asn  tyr  leu  phe  ser
CCTCCTTGCCCCAAGAACTACCTGTTCAGC
241                                           270
 cys  gln  pro  asn  leu  ala  pro  val  pro  cys
TGCCAGCCCAACCTGGCCCCAGTGCCATGC
271                                           300
 ser  ala  pro  arg
AGCGCCCCGAGA
301            312
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Fig. 5. Base sequence of the coding strand of the *Drosophila* insert in DmcMM99. The sequence is based on the results from sequencing the entire 312-bp insert in both directions using M13mp8 and M13mp9. Since the Northern data (Figure 1) suggest that the mRNA for DmcMM99 is ~650 bases long, this sequence represents approximately one-half of the message. The ATG at position 25 may be the initiating codon (underlined), but since the open reading frame continues 5′ to this location we have assumed for the purposes of translation product analysis that it is not. In general the sequence could be determined unambiguously with one possible exception. Although the cytidine residue at position 182 was clearly in evidence in six cases using M13mp9, evidence for its existence was weaker when using M13mp8. We have chosen to include this residue in this figure since the majority of the data supports its existence. However, even if our assumptions for data interpretation are incorrect, neither the exclusion of the first 24 bases from the translation product nor the altered reading frame from position 182 onward significantly affects the sequence results in terms of the argument we wish to make. The DmcMM99 translation product remains unusually high in proline and alanine.

probe under conditions of high stringency revealed a single region of hybridization (cr1 in Figure 7). Using lower criteria (Figure 8), the same probe also hybridized to a second region (cr2 in Figure 7). DmcMM99 did not hybridize to λLS1 at all under high criteria; however, under lower criteria, DmcMM99 hybridized with both regions, though more intensely with cr1 (data not shown).

Since DmcMM115 hybridized primarily with the 26A band on the second chromosome, we expected λLS1 to be derived from that site; however, it was possible that λLS1 could have originated from one of the chromosomal regions (32EF, 34C) which cross-hybridize with DmcMM115. We confirmed that λLS1 was indeed derived from region 26A on 2L by *in situ* hybridization using λLS1 as probe; λLS1 hybridized primarily with that site (Figure 6c). All of the above results are consistent with the interpretation that at least two VM genes are clustered at site 26A with each hybridizing region representing a different transcription unit: region cr1 corresponding to DmcMM115 and region cr2 corresponding to another member of the DmcMM99 family which remains to be identified.

Table I. Comparison of amino acid content of *Drosophila* vitelline membrane and DmcMM99 translation product

Amino acid	% in VM[a,b]	% from DmcMM99[a]
Ala	28.6	21.1
Pro	18.3	16.3
Ser	16.9	10.6
Gly	10.4	6.7
Asp + Asn	4.2	5.8
Leu	3.4	5.8
Tyr	3.5	4.8
Lys	3.0	4.8
Ile	1.3	4.8
Cys	1.0	4.8
Val	3.0	3.8
Glu + Gln	4.4	2.9
Phe	1.8	2.9
Met	0	2.9
Arg	0	2.9
Thr	0.9	1.0
His	0.4	1.0

[a]Residues/100 residues.
[b]Taken from Petri *et al.* (1976). Note that these figures represent the average for the total VM which is composed of ~10 different proteins.

Discussion

Both DmcMM99 and DmcMM115 represent relatively high abundance, ovary-specific transcripts which are not part of the maternal message pool (Mindrinos, 1984). We have shown here that these transcripts are of a size and stage specificity expected for VM mRNAs. Additionally, in support of this conclusion, we have shown that the information encoded by these clones specifies products whose size and amino acid content is consistent with VM precursors. The fact that most VM proteins individually share the characteristically high proline and alanine content found for the VM as a whole, (Mindrinos, 1984), suggests that many VM genes may be members of a multigene family. Indeed, the nucleic acid sequences we have identified as putative VM genes do appear to fulfill the criteria of a multigene family.

Under relatively low stringency, DmcMM99 and DmcMM115 are seen to cross-hybridize to common additional RNA species (Figure 1) which have stage-specific transcription patterns indicative of VM transcripts (Figure 2). The data from *in situ* hybridization show that this multiple band hybridization pattern is unlikely to represent differential splicing given the large distances between the chromosomal locations involved (Figure 5). In addition, the cross-hybridizing second 'transcription unit' we have identified in the λLS1 genomic clone may be evidence of another related VM gene and indicates that at least some clustering of VM genes may occur. However, in this case, given their proximity, cr1 and cr2 may simply be two cross-hybridizing exons of the same gene. Overall, our data suggest that VM genes are members of a multi-gene family of at least three or four members, all of which reside on the left arm of the second chromosome.

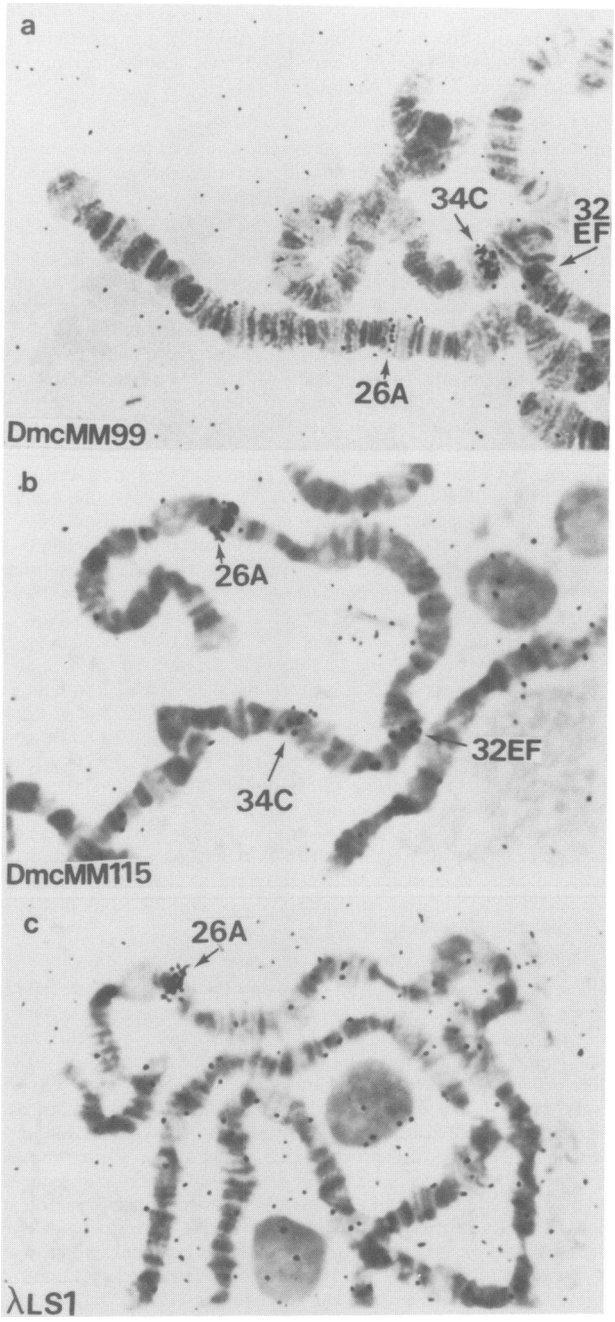

Fig. 6. *In situ* hybridization of recombinant clones. **(a)** DmcMM99; **(b)** DmcMM115 and **(c)** λLS1. Polytene band designations of regions of hybridization are indicated in each photograph.

Table II. Polytene band locations of major and minor sites of *in situ* hybridization using DmcMM99 and DmcMM115

Probe	Primary hybridization site	Secondary hybridization site
DmcMM99	34C	26A and 32EF
DmcMM115	26A	34C and 32EF

Since we expect the products from cell-free translation of mRNAs for secretory proteins to be larger than their processed counterparts, it is most likely that pVi represents the precursor for v10 or v11 and that pViii corresponds to the precursor for v14.5. Given the relative abundance of v11, we

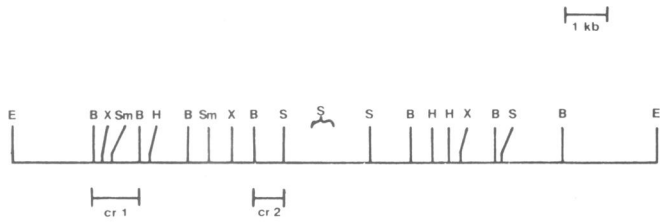

Fig. 7. Restriction map of λLS1. cr1 and cr2 designate regions complementary to a DmcMM115 probe. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sal*I; Sm, *Sma*I; X, *Xba*I.

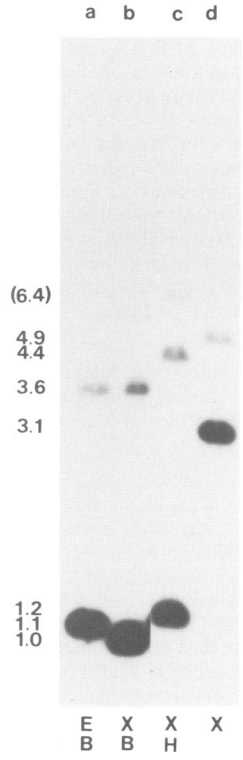

Fig. 8. Restriction fragments of λLS1 hybridized under low criteria with DmcMM115. When high stringency conditions are employed, only the restriction fragments containing cr1 (3.1 kb and less) hybridize to the probe (data not shown). Under the lower criteria conditions shown, additional hybridization to cr2 is observed as indicated by the relatively low intensity signal associated with fragments larger than 3.1 kb. The 6.4-kb fragment is an artifact of partial digestion. **(a)** *Eco*RI *Bam*HI digest, **(b)** *Xba*I *Bam*HI digest, **(c)** *Xba*I *Hind*III digest, **(d)** *Xba*I digest.

believe it is more likely that pVi is a precursor for this VM component. The nature of pVii is less clear. Given its position it could be a precursor for v13; however, its relative abundance could suggest that it may also be associated with v11 since v11 could contain more than one VM component or have more than one precursor form. Clearly, information from *in vitro* protein processing experiments, or partial proteolysis studies will be needed to clarify these associations.

Materials and methods

Drosophila stocks and clones

For all studies the Oregon-R strain of *D. melanogaster* was used as a source of RNA and eggshell proteins. Egg chambers were staged according to the criteria of King (1970). In several cases stages 10A and 10B were combined and called stage 10. DmcMM99 and DmcMM115 were derived from a cDNA library made by K.Jacobs and M.Mindrinos (Mindrinos, 1984 and Mindrinos *et al.*, in preparation). λLS1 was selected from a *Drosophila*, Oregon-R, 18 h

old embryo genomic library generously provided by J.Pustell (Harvard University).

RNA preparation

For obtaining specifically stage 8–10 egg chambers 1 day-old, post-eclosion females were used for dissection; otherwise, 2–3 day-old flies were used. Females were cold anesthetized and their ovaries hand dissected in ice-cold *Drosophila* Ringer's solution (Ephrussi and Beadle, 1936). After a maximum of 45 min, accumulated tissue was pelleted and stored at −80°C.

RNA was purified from frozen tissue generally following the method of Spradling and Mahowald (1979). Tissue was homogenized or vortexed briefly in lysis solution (10 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 1% SDS). Proteinase K was added and the mixture incubated at 37°C for 1–20 min. The sample was deproteinized by several extractions with two volumes of redistilled phenol:chloroform:isoamyl alcohol (25:24:1) (v/v/v). These extractions were repeated as many times as required to minimize the interphase formed after centrifugation. The RNA was then extracted twice with SEVAG (chloroform:isoamyl alcohol, 24:1) (v/v), adjusted to 0.3 M sodium acetate and precipitated with two to three volumes of cold, absolute ethanol. When appropriate, 10 μg yeast tRNA/ml of extract was added as carrier. Generally, RNA was allowed to precipitate at −20°C for at least 12 h and then collected by centrifugation at 15 000 *g* for 20 min at −5°C. Samples were washed with 70% ethanol, dried by vacuum and redissolved in distilled, deionized water and stored at −80°C. Such preparations were suficiently pure for gel electrophoresis and blot hybridization analysis. RNA for cell-free translation was purified further. RNA pellets were dissolved in 0.2 M NaOAc (pH 6), precipitated with ethanol, washed three times with 70% ethanol and redissolved in water. RNA was considered adequately purified when the ratio of optical densities at 260 nm/280 nm was 1.7–2.0.

Preparation of DNA

Plasmid DNA was prepared by the method of Birnboim and Doly (1979) as modified by D.Ish-Horowicz (see Maniatis *et al.*, 1982). Phage DNA was prepared essentially by the methods in Maniatis *et al.* (1982).

Electrophoresis and blotting of RNA

Denaturing 1.0–1.5% agarose gels containing 2.2 M formaldehyde were used for RNA blotting (Lebrach *et al.*, 1977). The gel and running buffers contained 20 mM MOPS (morphaline propane sulfonic acid) buffer, 5 mM NaOAc and 1 mM EDTA adjusted to pH 7 with NaOH (Lehrach *et al.*, 1977). The gel mixture was brought to 65°C and formaldehyde added last from a 13.2 M stock solution. RNA samples containing 50% formamide, 2.2 M formaldehyde and 1x running buffer were heated to 65°C for 5 min, chilled on ice, brought to final concentrations of 20% glycerol and 0.1% bromophenol blue and immediately loaded onto gels. To visualize the RNA pattern, a portion of the gel was equilibrated in water for 20–30 min and then stained with 1 μg ethidium bromide/ml for 10 min. Destaining in water usually required >1 h to sufficiently reduce background.

For hybridizations, unstained portions of gels were blotted onto nitrocellulose paper following the Thomas (1980) protocol. The paper was soaked in water at 50°C for 15 min and dipped in 20 x SSC (3 M NaCl, 0.3 M trisodium citrate) just before blotting. Transfers proceeded with 20 x SSC for at least 12 h using standard techniques (e.g., Maniatis *et al.*, 1982). The nitrocellulose was then baked in a vacuum oven at 80°C for 2–3 h. Prior to prehybridization the paper was soaked in 2 x SSC for 2–5 min.

Hybridization to RNA blots

Hybridization of DNA probes to the RNA blots followed the methods/conditions described by Wahl *et al.* (1979). The paper was placed in a heat-sealable boiling bag and pre-treated with a buffer (150 μl/cm²) containing 50% formamide, 0.1% SDS and 500 μg/ml heat-denatured, sheared calf thymus DNA at 42°C for several hours (usually overnight). The hybridization buffer contained 50% formamide, 4 x SSC, 4 x Denhardt's solution (Denhardt, 1966), 20 mM sodium phosphate (pH 6.4), 0.1% SDS and 500 μg/ml denatured, sheared calf thymus DNA. Hybridizations proceeded for 24–36 h using 300 μl of buffer for each cm² paper. DNA probes were prepared by nick-translation following the procedures of Maniatis *et al.* (1982) and Rigby *et al.* (1977) using a mixture of [^{32}P]dNTPs at 800 Ci/mM (New England Nuclear). 2.5–5.0 $(10)^5$ c.p.m. of heat-denatured DNA probe was used for each ml of buffer. Hybridization temperatures varied from 37°C to 45°C depending on the purpose of the experiment. Following hybridization the paper was washed twice with 2 x SSC, 0.1% SDS, 50% formamide at 42°C for 30 min each time and then once with 1 x SSC, 0.1% SDS, 50% formamide at 42°C for 1 h. The nitrocellulose was then covered with Saran wrap and autoradiographed using an intensifying screen at −80°C. Blots were reused twice after removal of the probe by boiling in water for 3 min.

Labelling and electrophoresis of proteins

Follicles and ovaries were dissected and cultured as described by Petri *et al.* (1979). [^{3}H]proline was purchased from New England Nuclear (NET-323). Samples were analyzed in Laemmli (1970) style SDS gels containing 15% acrylamide. Radioactive samples were detected by autofluorography using ENhance from New England Nuclear.

Cell-free translations

Translation of mRNA in a wheat germ system utilized material supplied by BRL; however, optimal reaction conditions for eggshell mRNA called for 3.5 mM Mg^+ and 100 mM K^+. 5 μCi of [^{3}H]proline was used as a tracer with 10 μg of total cytoplasmic RNA in a 30 μl reaction mix at 25°C for 90 min.

Hybrid selection

Recombinant plasmid DNA used for hybrid selections was spotted onto nitrocellulose filters according to a modification (Iatrou and Tsitilou, personal communication) of a technique described by Kafatos *et al.* (1979). The plasmid DNA was linearized, phenol extracted, and then denatured by incubation at room temperature for 10 min, with 0.3 N NaOH. The DNA solution was neutralized with one volume of 2 M ice-cold ammonium acetate and placed on ice. 25 mm nitrocellulose filter discs washed for 1 h with water were placed on top of two additional filters on a Millipore filtering apparatus. The filters are washed with ice-cold 1 M ammonium acetate and the 3 μg/clone of DNA spotted using a 100 μl Clay Adams micropipette during the application of mild suction. The filter was kept moist until all DNA was applied. The filters were then flooded with 1 M ammonium acetate and washed with ~100 ml of cold 4 x SSC passed through the filters with substantial suction. Clusters of dots corresponding to the same clone were then excised with a clean razor blade and baked for 2 h at 80°C.

Dots were pre-hybridized for 1 h at 37°C with a buffer containing 50% formamide, 0.6 M NaCl, 100 mM Pipes pH 6.5 and 100 μl/ml poly(A). Hybridization was carried out in a 30 μl volume at 37°C for 2 h with the same buffer, but replacing poly(A) with the RNA sample to be used for hybrid selection. The amount of RNA was such that there was a 10-fold excess of DNA. Filters were washed 8 x with 1 x SSC, 0.1% SDS at 65°C by vortexing briefly and decanting (Ricciardi *et al.*, 1979). Finally, the filters were washed twice with 10 mM Tris pH 7.5, 2 mM sodium EDTA at room temperature. For elution of the RNA, 100 μl of water are added and the hybridized RNA released by incubation for 1 min at 100°C. After quick freezing in dry ice-ethanol, eluted RNA samples were transferred to new tubes, ammonium acetate added to a final concentration of 0.3 M and the RNA precipitated with ethanol and carrier yeast tRNA.

M13 cloning, DNA sequencing and sense strand determination

Initially sequencing was done using pUC9 directly; however, improved resolution was obtained when the *Drosophila* insert was transferred to M13 vectors. *Eco*RI-*Hind*III fragments used for sequencing were purified from low melting temperature agarose after gel electrophoresis using the ELU-tip method (Schleirer and Schuell), and subcloned in the appropriate restriction sites of M13mp8 and M13mp9. M13 clones and single-stranded DNA templates were prepared as described by Messing (1983) and Messing and Vieira (1983). Nucleotide proteins were determined by the dideoxy nucleotide chain termination method of Sanger *et al.* (1977), with 0.4 mm sequencing gels as modified by Garoff and Ansorge (1981). All sequencing reagents were obtained from Boehringer Mannheim Biochemicals. In order to determine which strand represented the sense strand, filters containing total ovarian RNA were hybridized with ^{32}P-labelled single-stranded M13mp8 and M13mp9 probes as described by Hu and Messing (1982). Only the single-stranded M13mp9 clone contained insert DNA that hybridized to blotted RNA.

In situ hybridization

Salivary gland squashes were prepared as described by Pardue and Gall (1975), heat-treated at 70°C (Bonner and Pardue, 1976), acetylated (Hayashi *et al.* (1978) and base denatured (Pardue and Gall, 1975). Radioactive probes were prepared by nick-translation essentially following the method of Maniatis *et al.* (1982) using [^{3}H]dCTP and [^{3}H]TTP. Ethanol precipitated probes were dissolved in 50% formamide and 2 x SSCP, pH 7.0, denatured at 85°C for 10 min, and applied to squashes (8 μl containing 200 000 c.p.m./squash) and hybridized for 24 h at 37°C. Squashes were washed in 2 x SSC once for 10 min at room temperature, then 3 x at 60°C for 10 min each and finally once again at room temperature for 15 min. Slides were processed for autoradiography as decribed by Pardue and Gall (1975) except that ammonium acetate was added to the Kodak NTB-2 emulsion at a final concentration of 300 mM (Brahic and Haase, 1978).

Selection and characterization of genomic clone λLS1

The genomic clone λLS1 was selected and purified from a bacteriophage lambda library by *in situ* plaque hybridization (Benton and Davis, 1977), using DmcMM115 as a probe. The library was constructed by partial *Sau*3A diggestion of Oregon-R genomic DNA and ligation into the *Bam*HI site of the

vector EMBL4. Average insert length was 14 kb. The restriction map of λLS1 was determined by analysis of single and double digests on 0.4 – 1.5% agarose gels.

Southern analysis of λLS1

Electrophoresed digests of λLS1 were transferred to nitrocellulose and hybridized with nick-translated DmcMM99 or DmcMM115 using the method of Southern (1975). Two different wash conditions were employed: relatively high criteria conditions consisted of two, 15 min washes at room temperature in 2 x SSC, 0.2% SDS followed by two, 30 min washes in 0.2 x SSC, 0.2% SDS, the first at room temperature and the second at 68°C; relatively low criteria conditions were identical except that the last two washes were both in 1 x SSC, 0.2% SDS.

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Note added in proof

A recent paper by Higgins *et al. (Dev. Biol.,* **105**, 155-165, 1984) also characterizes a clone similar to λLS1. Our results are consistent with theirs.