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Infection of the germ line by retroviral particles produced in the follicle cells: a possible mechanism for the mobilization of the *gypsy* retroelement of *Drosophila*

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SUMMARY

The *gypsy* retroelement of *Drosophila* moves at high frequency in the germ line of the progeny of females carrying a mutation in the *flamenco* (*flam*) gene. This high rate of *de novo* insertion correlates with elevated accumulation of full-length *gypsy* RNA in the ovaries of these females, as well as the presence of an *env*-specific RNA. We have prepared monoclonal antibodies against the *gypsy* Pol and Env products and found that these proteins are expressed in the ovaries of *flam* females and processed in the manner characteristic of vertebrate retroviruses. The Pol proteins are expressed in both follicle and nurse cells, but they do not accumulate at detectable levels in the oocyte. The Env proteins are expressed exclusively in the follicle cells starting at stage 9 of oogenesis, where they accumulate in the secretory apparatus of the endoplasmic reticulum. They then migrate to the inner side of the cytoplasmic membrane where they assemble into viral particles. These particles can be observed in the perivitelline space starting at stage 10 by immunoelectron microscopy using anti-Env antibodies. We propose a model to explain *flamenco*-mediated induction of *gypsy* mobilization that involves the synthesis of *gypsy* viral particles in the follicle cells, from where they leave and infect the oocyte, thus explaining *gypsy* insertion into the germ line of the subsequent generation.

Key words: *Drosophila*, retroelement, retrovirus, oogenesis, *flamenco*, *gypsy*, germ line, follicle

INTRODUCTION

The *gypsy* retroelement of *Drosophila melanogaster* displays striking similarities in its structure and organization to the proviral form of vertebrate retroviruses. *Gypsy* is flanked by two long terminal repeats (LTRs) and, unlike most retroelements, contains three open reading frames (ORFs) (Freund and Meselson, 1984; Marlor et al., 1986). The first and second open reading frames encode proteins homologous to the Gag and Pol products of retroviruses. In addition, ORF3 is expressed from a spliced message that encodes a protein containing a signal peptide, endopeptidase cleavage site and glycosylation sites, all characteristic of retroviral Env proteins (Pélisson et al., 1994). A similar structural organization is found in the *tom* element of *Drosophila ananassae* (Tanda et al., 1994). These similarities have prompted the suggestion that *gypsy* and other ORF3-containing retroelements represent endogenous insect retroviruses (Boeke 1988; Boeke and Corces 1989; Coffin 1993).

The *gypsy* retroelement moves unpredictably and at low frequency in the genome of the species that it populates, but some strains have been described in which *gypsy* movement occurs with high frequency (Kuhn 1970; Laverty and Lim 1982; Gerasimova et al., 1984a,b). More recently, Mével-Ninio et al. (1989) have identified a strain in which *gypsy* insertion into the X-linked *ovo* gene takes place at frequencies of up to 1.5×10^{-1}. Mobilization of *gypsy* in this particular strain depends on the presence of a mutation in the *flamenco* (*flam*) gene (Prud'homme et al., 1995). This mutation is also responsible for *gypsy* mobilization in a different genetically unstable mutator strain (Kim et al., 1990; Kim and Belyaeva, 1991). *flam* mutations show a typical maternal effect on *gypsy* mobilization: new insertions are observed in the progeny of homozygous *flam* mothers but not in descendants of heterozygous females. The frequency of new *gypsy* insertions in this strain can be determined by measuring the reversion rate of the dominant female sterile allele ovo^D (Mével-Ninio et al., 1989). Revertants of this mutation are caused by insertion of *gypsy*, and occur at frequencies between 1 and 15% depending on the age of the females: newly eclosed females give rise to progeny with a high ovo^D reversion rate and the frequency of this event decreases as the females age (Prud'homme et al., 1995).

Mobilization of *gypsy* in the progeny of *flam* females correlates with higher accumulation levels of the full-length *gypsy* RNA in the ovaries of these flies. In addition, mutations in the *flam* gene result in the synthesis of a novel transcript as a consequence of a splicing event between a donor site located in

gag and an acceptor site located at the beginning of ORF3. This spliced RNA accumulates specifically in the follicle cells of *flam* female egg chambers (Pélisson et al., 1994) and encodes a protein that is expressed in the same cells and has many of the properties expected for a retroviral Env protein (Song et al., 1994). Retroviral-like particles have been isolated from ovary extracts obtained from *flam* homozygous females, but not from heterozygous or wild-type strains. These particles react with *gypsy* ORF3 antibodies, suggesting that they correspond to *gypsy* retroviruses. Their infectivity has been demonstrated by experiments in which either purified particles (Song et al., 1994) or crude extracts (Kim et al., 1994) were fed to *Drosophila* larvae from a strain (SS) lacking active copies of *gypsy*; new sites of *gypsy* insertion were then observed in the progeny of the fed flies by in situ hybridization to polytene chromosomes or by genetic means using the ovo^D reversion assay. *Gypsy* mobilization was inhibited by preincubation of purified particles with antibodies against *gypsy* Env, suggesting that *de novo* insertion of the provirus requires retroviral particle infectivity (Song et al., 1994).

The question remains as to how mutations in the *flam* gene in females gives rise to *gypsy* mobilization in their progeny. Accumulation of both *gypsy* full-length RNA and Env protein has been detected in the follicle cells of *flam* females at stage 10 of oogenesis (Pélisson et al., 1994), but these cells are shed after forming the chorion. A mechanism must then exist to transfer *gypsy*-encoded materials from the somatic follicle cells to the germ line, to ensure their presence in the next generation. The formation of *gypsy* infectious particles could provide such a mechanism if synthesis of other viral components such as Gag and Pol products also occurs in the ovaries of *flam* females, and if expression of these proteins and assembly of viral particles takes place before deposition of the vitelline membrane during stage 10 of oogenesis could interfere with oocyte infection. Here, we have analyzed the expression of *gypsy*-encoded proteins to explore how *gypsy* is transmitted between generations. Using monoclonal antibodies raised against specific domains of the Env protein, we present evidence suggesting that *gypsy* Env is processed into surface and trans-membrane polypeptides, similar to vertebrate retrovirus Env proteins. Expression of *pol*-encoded proteins is explored for the first time and can also be observed in ovaries of *flam* females. Pol protein synthesis, like Env synthesis, is increased as a consequence of the *flam* mutation. Finally, we have visualized assembly of *gypsy* particles by electron microscopy in the follicle cells of flies carrying the *flam* mutation and these observations provide the basis for a novel model to explain how *gypsy* is transmitted from generation to generation. The *gypsy* virions appear to move through the perivitelline space during a brief developmental window of opportunity and infect the oocyte, providing a mechanism to explain *gypsy* insertion in the next generation.

MATERIALS AND METHODS

Strains and genetic crosses

The strains MG#3 (*y v f mal flam/FM3)* (Prud'homme et al., 1995), ovo^{D1} *v* (Mével-Ninio et al., 1989) and SS (*w flam*) (Kim et al., 1990) were provided by A. Bucheton. These strains are maintained on standard *Drosophila* medium; all genetic experiments were carried out at 25°C.

Generation of monoclonal antibodies

The monoclonal antibodies 7B3, 8E7 and 8H10 were generated using the following protocol. A 1612 bp *Sty*I-*Xho*I *gypsy* fragment was inserted into pATH3 (Koerner et al., 1991) for TrpE-ORF3 fusion protein production. A 1437 bp *Sty*I fragment was cloned into the pATH1 vector for production of a TrpE-ORF2 fusion protein. Cells containing both constructs were induced with β-indole acrylic acid and 89 kDa TrpE-ORF3 and 82 kDa TrpE-ORF2 fusion proteins were isolated. BALB/c mice (5-6 months old) were immunized with the fusion proteins. Mice received an initial injection of 100 μg of protein emulsified 1:1 with Freund's complete adjuvant. After 2 weeks, the mice were given three boosts of 100 μg of protein in Freund's incomplete adjuvant at 2 week intervals. 6 days after the final boost, serum samples were tested by immunoblotting. Mice giving good serum responses were boosted with 100 μg of protein with Freund's incomplete adjuvant 4 days before the fusion. Spleen cells were fused in the presence of PEG 4000 (GIBCO) to sp2/0 myeloma cells, using standard protocols (Harlow and Lane, 1988). Hybridoma supernatants were screened 1-2 weeks later on immunoblot strips containing either TrpE or the fusion protein. Antibodies 7B3 and 8E7 react only with the TrpE-ORF3 fusion protein, whereas antibody 8H10 reacts exclusively with the TrpE-ORF2 fusion product. Positive preclones were cloned by limiting dilution.

Western analysis

Ovaries from 3- to 5-day-old female flies were isolated in buffer (0.1 M NaCl, 0.01 M Tris-HCl pH 7.4, 0.001 M EDTA, 0.001 M PMSF) and transferred into SPS lysis buffer (2.5% SDS, 60 mM Tris-HCl pH 7.4, 0.005% bromophenol blue, 10% glycerol) for homogenization. After homogenization, proteins were boiled for 10 minutes, spun for 5 minutes, and stored at –20°C. Gradient fractions (100 μl) were precipitated by 10% TCA and resuspended in sample buffer. Samples for immunoblotting were prepared as above. Proteins were run on 12% polyacrylamide gels and electroblotted onto nitrocellulose. Immunoblots were blocked for 0.5-1 hour with 5% powdered milk in PBST (150 mM NaCl, 10 mM phosphate pH 7.0, 0.3% Tween 20). Primary antibodies (hybridoma culture supernatant diluted 1:10) were added in 1% milk in PBST and incubated for 1.5 hours at room temperature. Blots were washed for 1 hour in PBST and incubated with peroxidase-conjugated secondary antibodies (Sigma) for 1.5 hours in PBST at a dilution of 1:10000. The washing procedure was repeated and the blots were subjected to ECL western blotting protocols (Amersham).

Whole-mount immunocytochemistry

Ovaries were dissected in BSS/PMSF (15 mM Tris-HCl pH 6.95, 50 mM NaCl, 40 mM KCl, 7 mM $MgSO_4$, 5 mM $CaCl_2$, 20 mM glucose, 1 mM PMSF) and fixed in 2% formaldehyde in PEM/NP40 (0.1 M Pipes pH 6.95, 2 mM EGTA, 1 mM $MgSO_4$, 1% NP40) for 30 minutes at room temperature. The fixative was removed by rinsing three times with antibody wash (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP40) and blocked for 45-60 minutes at room temperature in antibody block (antibody wash plus 5% dry milk). The primary antibody was applied as 1:200 dilution in antibody wash plus 1% dry milk and incubated with ovaries overnight at 4°C. After six changes of antibody wash during a 60 minute period, ovaries were blocked in antibody block for 30 minutes at room temperature and incubated with secondary antibody labeled with fluorescein (Cappel) in antibody wash for 2 hours at room temperature. The secondary antibody had been preabsorbed against ovaries overnight at 4°C and diluted 1:200 in antibody wash before use. After incubation with secondary antibody, ovaries were washed with six changes of antibody wash for 1 hour at room temperature. Ovaries were mounted in 70% glycerol/0.01% paraphenylene diamine and observed using a Bio-Rad MRC 600 confocal microscope.

Electron microscopy

Electron microscopy of thin sections was carried out as follows.

Ovaries were dissected in BSS/PMSF and fixed in 4% formaldehyde in PEM buffer for 30 minutes as described above. These ovaries were then fixed in 4% glutaraldehyde in calcium cacodylate pH 7.4, and subsequently in 1% osmium tetroxide, 0.5% KFeCN in calcium cacodylate. Ovaries were then embedded in agarose and stained in 0.5% uranyl acetate. After overnight staining, ovaries were embedded in epoxy resin and cut into 85 nm sections using an ultramicrotome. Sections were then examined using a Joel 100S electron microscope.

Immunogold labeling for electron microscopy

Ovaries were dissected in BSS/PMSF and fixed for 30 minutes at room temperature in 4% paraformadehyde/PBS. After rinsing in 0.1 M $NaPO_4$, 3.5% sucrose (PS) three times 5 minutes each, ovaries were incubated in 0.25% tannic acid for 1 hour, rinsed in PS three times 5 minutes each, and then rinsed in 0.1 M maleate, 4% sucrose (MS) three times 5 minutes each. Ovaries were then stained in 2% uranyl acetate in MS, rinsed three times in MS 5 minutes each, dehydrated and embedded in LR white resin (Polyscience). Sections (90 nm) were dried on nickel grids and subjected to the following procedure. Grids were blocked in TBST (10 mM Tris-HCl pH 7.2, 500 mM NaCl, 0.05% Tween 20)/1% BSA for 15 minutes and incubated with the primary antibody in TBST/1% BSA at 4°C overnight; primary antibodies had been previously purified using the mAb TrapII kit from Pharmacia. Grids were then rinsed on 5 drops of TBS and incubated on a drop of gold-labeled goat anti-mouse IgG (Amersham) at a concentration of 1:30 for 1 hour. Grids were rinsed on 3 drops of TBS and then on 2 drops of H_2O, fixed in 2.5% glutaraldehyde for 5 minutes and rinsed on 3 drops of H_2O for 1 hour. Grids were then stained in 2% OsO_4 for 15 minutes, washed on 3 drops of H_2O and dried. Electron microscopy was performed on a Zeiss TEM 10A transmission electron microscope.

In vitro transcription/translation

A 1.5 kb DNA fragment, corresponding to the spliced ORF3 mRNA, was amplified using primers 1463 (5′-ACGAAGCAATACATTGTTAGTTGT-3′) and 1475 (5′-AGTTAAGTTAGAAAAGCATGTTCACCCTCATGATGTTCATACCCTTG-3′). These PCR fragments were directly cloned into the TA cloning vector (pCRII, Invitrogen Corp.) to give rise to plasmid p6. The orientation of the insert was determined by restriction enzyme digestions and sequencing. Plasmid p6 has *gypsy* ORF3 under the control of the bacteriophage T7 promoter. Plasmids p2 and p5 were constructed by digesting p6 with the restriction enzymes PpumI and PflmI respectively, followed by religation of the linear DNAs. Plasmids p3 and p4 were constructed by amplifying the surface and trans-membrane coding sequences using two specific primer sets: primers 1475 and 1734 (5′-TTATTAGCGCCGAGACCGCTCGC-3′) for surface and primers 1735 (5′-ATGATGGAAACTTGCGTGCGCTC-3′) and 1463 for trans-membrane. Coupled in vitro T7 transcription-translation was done with the TnT-coupled reticulocyte lysate system (Promega) following their standard protocol. For the production of non-radiolabeled protein, both amino acid mixture (-Met) and amino acid mixture (-Leu) were used in the reaction.

RESULTS

Gypsy ORF3 is processed into surface and trans-membrane proteins

The *gypsy env*-specific spliced RNA encodes a putative protein of 54 kDa; if this protein is processed at a putative endopeptidase cleavage site, it would give rise to surface and trans-membrane proteins of 32 kDa and 20 kDa respectively (see below). We have previously reported that *gypsy* ORF3 is expressed into several polypeptides in the ovaries of females carrying the *flam* mutation (Song et al., 1994). To determine the nature of these different proteins, we prepared monoclonal antibodies against a TrpE-ORF3 fusion protein. Two different monoclonal antibodies were obtained that give rise to distinct staining patterns when used on western blots containing protein extracts from ovaries of *flam* females (Fig. 1). One of these monoclonal antibodies, named 7B3, has been previously described; it detects proteins with sizes of 66 kDa, 54 kDa, and 28 kDa (Song et al., 1994). A newly isolated antibody named 8E7 detects the 66 kDa and 54 kDa proteins in common with 7B3, but fails to recognize the 28 kDa protein, immunoreacting with a 34 kDa polypeptide instead (Fig. 1). All these proteins are specifically expressed in ovaries of homozygous *flam* females but do not accumulate at detectable levels in heterozygotes or in flies from the SS strain that lacks active copies of *gypsy*.

To gain further insights into the nature of these different polypeptides, we mapped the epitopes on the Env protein recognized by these two monoclonal antibodies. Plasmids encoding the full-length Env protein (p6), the putative surface and trans-membrane polypeptides (p3, p4), or other proteins of various sizes (p2, p5) (Fig. 2A), were made and used in a combined in vitro transcription-translation system to synthesize these different proteins in the presence of ^{35}S-Met (Fig. 2B). The products of these reactions were then subjected to western analysis using the 7B3 (Fig. 2C) and 8E7 (Fig. 2D) antibodies. Both antibodies recognized the proteins expressed from plasmid p6, which encodes the full-length Env protein, and plasmid p5, which encodes a protein lacking the carboxy-terminal end of the trans-membrane protein. In addition, 8B7 but not 7B3 recognizes two polypeptides synthesized in vitro at very low levels from plasmid p4, which encodes the putative trans-membrane protein. In contrast, 7B3 but not 8E7

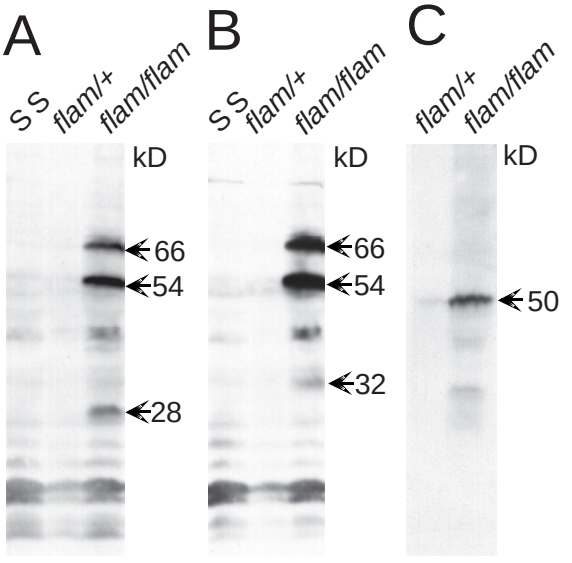

Fig. 1. Expression of Env and Pol proteins in the ovaries of *flam* and wild-type strains. Western analysis of proteins extracts from ovaries of *flam*/*flam* homozygotes, *flam*/+ heterozygotes, and the SS wild-type strain, which lacks active *gypsy* elements. Protein extracts (70 μg) were run on a 12% SDS-PAGE gel and immunoblotted with either Env monoclonal antibodies 7B3 (A), 8E7 (B), or anti-IN monoclonal antibody 8H10 (C). Proteins present in *flam* ovaries but missing in the SS strain are marked by arrows. Sizes in kDa are indicated on the right side of each panel.

recognize a polypeptide synthesized from plasmid p3, which encodes the putative surface protein. Finally, neither antibody recognized a polypeptide made from plasmid p2 encoding a truncated surface protein that lacks the carboxy-terminal end. These results suggest that monoclonal antibody 7B3 recognizes an epitope located in the carboxy-terminal part of the putative surface protein, and monoclonal antibody 8E7 specifically reacts with sequences present in the putative trans-membrane region of ORF3.

The pattern of *env*-encoded proteins present in ovaries of *flam* females (Fig. 1) can now be interpreted in the context of the specificity of these two monoclonal antibodies. The 64 kDa and 45 kDa proteins are both recognized by 7B3 and 8E7, suggesting that they correspond to unprocessed precursors of the mature Env products. The 66 kDa protein might correspond to the glycosylated form of the full-length Env protein including the signal peptide; the precise structure of the 54 kDa protein is not yet understood. Finally, the 28 kDa protein recognized specifically by 7B3 probably corresponds to the processed surface protein, whereas the 34 kDa polypeptide that reacts with 8E7 must correspond to the trans-membrane protein. These results indicate that the envelope protein encoded by *gypsy* is processed in the manner required for its functional activation and that the mature components of *gypsy* Env accumulate in the ovaries of *flam* females where they could be assembled into infectious retroviral particles.

Pol proteins are expressed in the ovaries of *flam* females

To test whether other components necessary for the synthesis of *gypsy* viral particles were also expressed in ovaries of *flam* females, we prepared monoclonal antibodies against a TrpE-Pol fusion protein containing 300 bp of the reverse transcriptase and most of the integrase coding regions. In vertebrate retroviruses, Pol is expressed as a Gag-Pol polyprotein that is processed by retroviral proteinases to give rise to a mature protease, reverse transcriptase/RNaseH and integrase proteins by proteolytic cleavage. The predicted size of the *gypsy* Gag-Pol precursor protein is 166 kDa. If Pol is processed in the same manner as in vertebrate retroviruses it would give rise to a ca. 60 kDa reverse transcriptase protein and a ca. 40 kDa integrase. One of the monoclonal antibodies obtained, named 8H10, recognizes a major protein of 50 kDa that is present in the ovaries of *flam* females but is absent from *flam*/+ fly ovaries (Fig. 1C); this protein is also absent in the ovaries of the SS strain (data not shown). Epitope mapping experiments similar to those carried out for Envelope indicate that monoclonal antibody 8H10 recognizes an epitope present in the Integrase (IN) coding region (data not shown). These results indicate that *pol*-encoded proteins are expressed and processed in the ovaries of *flam* females and, therefore, other components of *gypsy* viral particles in addition to Env are present in the ovary and are available for the assembly of mature *gypsy* retrovirus particles.

Expression of Pol and Env proteins during *Drosophila* oogenesis

Western analyses of protein extracts indicate the presence of IN and Env proteins in the ovaries of *flam* females. Further-

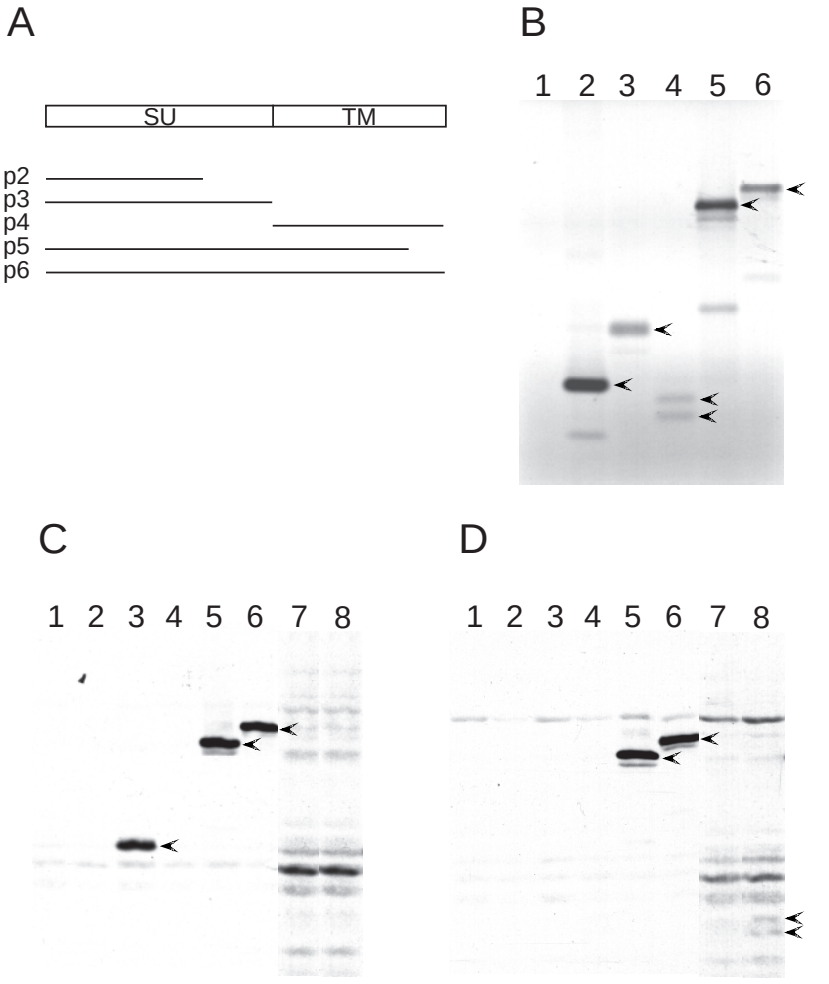

Fig. 2. Epitope mapping of anti-Env monoclonal antibodies. (A) Structure of the Env protein with a vertical line indicating the endopeptidase cleavage site separating the putative surface (SU) and trans-membrane (TM) polypeptides. Horizontal lines below indicate the extent of cloned fragments used for in vitro transcription-translation; numbers on the left side of each line indicate the name assigned to each plasmid. The inferred epitope locations for mAbs 7B3 and 8E7 are indicated by arrows above the diagram of the Env ORF. (B) ^{35}S-Met-labeled in vitro translation products were run on a 12% SDS-PAGE and the gel was subjected to autoradiography to visualize labeled proteins. Lane 1 contains in vitro translated proteins with no DNA added. Numbers on top of other lanes correspond to proteins translated from the same number plasmid indicated in A. Arrowheads indicate the major proteins in the translation reaction that correspond in size to the expected products encoded by the respective plasmids. (C) Non-radioactive translation products from the same plasmids as in B subjected to western analysis using antibody 7B3; lane 7 is a longer exposure of lane 1, whereas lane 8 is a longer exposure of lane 4. (D) Same as in C except that antibody 8E7 was used in the western analysis; lane 7 is a longer exposure of lane 1, whereas lane 8 is a longer exposure of lane 4. Arrowheads in C and D indicate the major products detected by the antibodies that correspond in size to those observed in B.

more, the *gypsy env*-specific RNA and encoded proteins accumulate specifically in the follicle cells during late stage 10 of oogenesis (Pélisson et al., 1994). At this time, the vitelline membrane is already in place, forming an impenetrable barrier for putative infectious particles present in the follicle cells. To further understand the relationship between *gypsy* expression, production of infectious particles and *gypsy* mobilization, we examined the pattern of spatial and developmental accumulation of IN and Env proteins during different stages of oogenesis in wild-type and *flam* females. The purpose of this analysis was to determine whether components of *gypsy* viral particles were present in the follicle cells at early stages of oogenesis, before formation of the vitelline membrane at stage 10B could interfere with their transfer to the oocyte. *Gypsy* Env proteins are expressed in the follicle cells at very low levels during stages 1 through 8 of oogenesis (Fig. 3A), and their synthesis increases appreciably during stage 9 (Fig. 3B). Accumulation of Env protein is highest during stages 10A and 10B (Fig. 3C,D), and it is present in the nurse cell-associated follicle cells as well as those surrounding the oocyte; expression seems to be highest in the centripetal cells that move anteriorly and surround the oocyte during stage 10. High levels of expression in these cells persist through stage 11, with much lower levels in the follicle cells adjacent to the posterior end of the oocyte (Fig. 3E). As the follicle cells secrete the chorion and degenerate during stages 12 through 14, the presence of Env protein is limited to the most anterior follicle cells (Fig. 3F-H). It is important to note that Env expression is limited to *flam* female egg chambers; it is undetectable in ovaries of the SS strain, which lacks active copies of *gypsy* (Fig. 3I,J), and is only detectable during stage 10 in the anterior follicle cells in *flam*/+ heterozygotes (Fig. 3L) but not at earlier stages (Fig. 3K).

Expression of Integrase follows a similar temporal pattern but it is not limited to follicle cells. Integrase protein is only detectable at background levels during stages 1-8 of egg chamber development (Fig. 4A) and its expression increases during stage 9 (Fig. 4B). Accumulation of Integrase at this and subsequent stages takes place in both nurse cells and follicle cells. The levels of Integrase protein are highest during stage 10 (Fig. 4C-E), and then decrease as the nurse cells degenerate and dump their contents into the oocyte (Fig. 4F-H). Interestingly, Integrase protein does not accumulate at detectable levels in the oocyte. This is also true for *gypsy* full-length RNA (Smith and Corces, 1995) and it is probably due to the short life of Pol proteins that do not assemble into viral particles. As with Env proteins, Integrase is not detectable in the wild-type SS strain (Fig. 4I-J), and is present at very low levels in egg chambers of *flam*/+ heterozygous females (Fig. 4K-L).

Gypsy viral particles are produced by follicle cells of *flam* females

Both Pol and Env proteins are expressed in follicle cells of *flam* egg chambers starting at stage 9 of oogenesis before vitelline membrane formation. Since Gag gene products are expressed as a Gag-Pol polyprotein, Gag proteins must also be present in the same cells at this stage of development. Thus, all the components necessary for the assembly of complete *gypsy* retroviral particles are available within a single type of cell, namely the follicle cells. To determine whether *gypsy* virions are actually present in follicle cells, we followed the subcellular distribution of Env protein during *Drosophila* oogenesis using immunoelectron microscopy. During stage 9 of egg chamber development, all the Env protein accumulates in the endoplasmic reticulum of the follicle cells (Fig. 5A). By stage 10A, the Env protein has migrated to the vicinity of the cell membrane where it can be observed in small patches regularly distributed throughout the outer cytoplasm (Fig. 5B). By stage 11, the perivitelline membrane separating the follicle cells and the oocyte has formed. Env immunoreactive material is much less abundant; instead, virus-like spherical structures that cross-react with anti-Env antibodies can be detected embedded in the

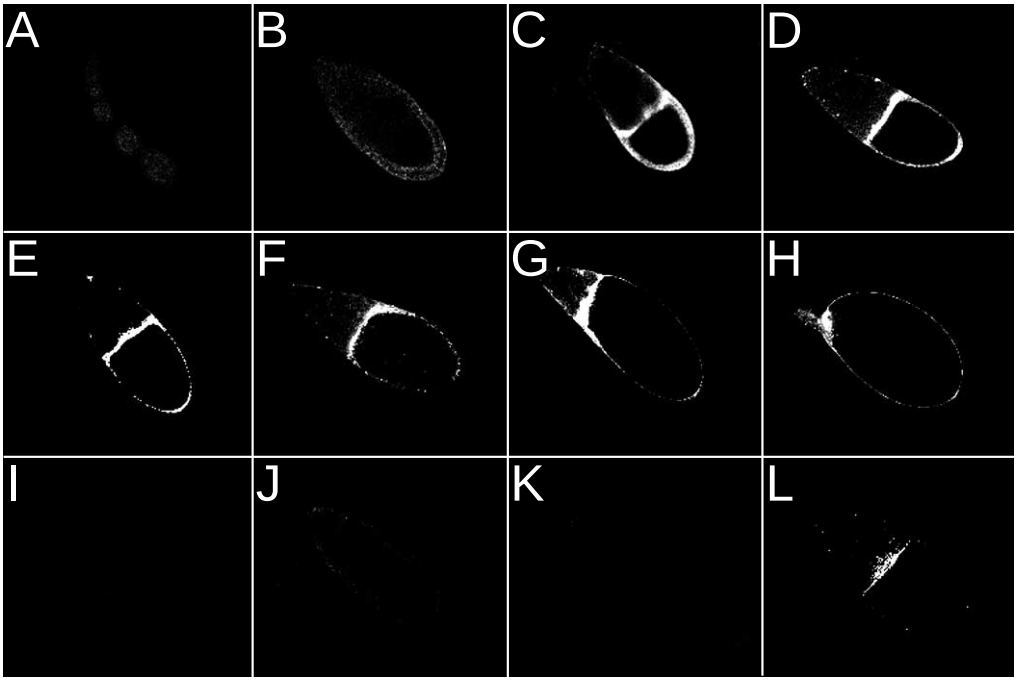

Fig. 3. Expression of Env proteins during *Drosophila* oogenesis. Immunoflurescence analysis using confocal microscopy of *gypsy* Env proteins using monoclonal antibody 7B3; antibody 8E7 gives the same pattern of staining. (A) Stages 1-8 egg chambers from females carrying the *flam* mutation; low level staining can be observed at this time of development; (B) stage 9 egg chambers of *flam* ovaries showing initial expression levels of Env proteins; (C) stage 10A; (D) stage 10B; (E) stage 11; (F) stage 12; (G) stage 13; (H) stage 14; (I) stages 1-9 of egg chamber development in ovaries from strain SS females; (J) stage 10 of oogenesis in SS females; (K) stages 1-9 of oogenesis in *flam*/+ females; (L) stage 10 *flam*/+ egg chamber.

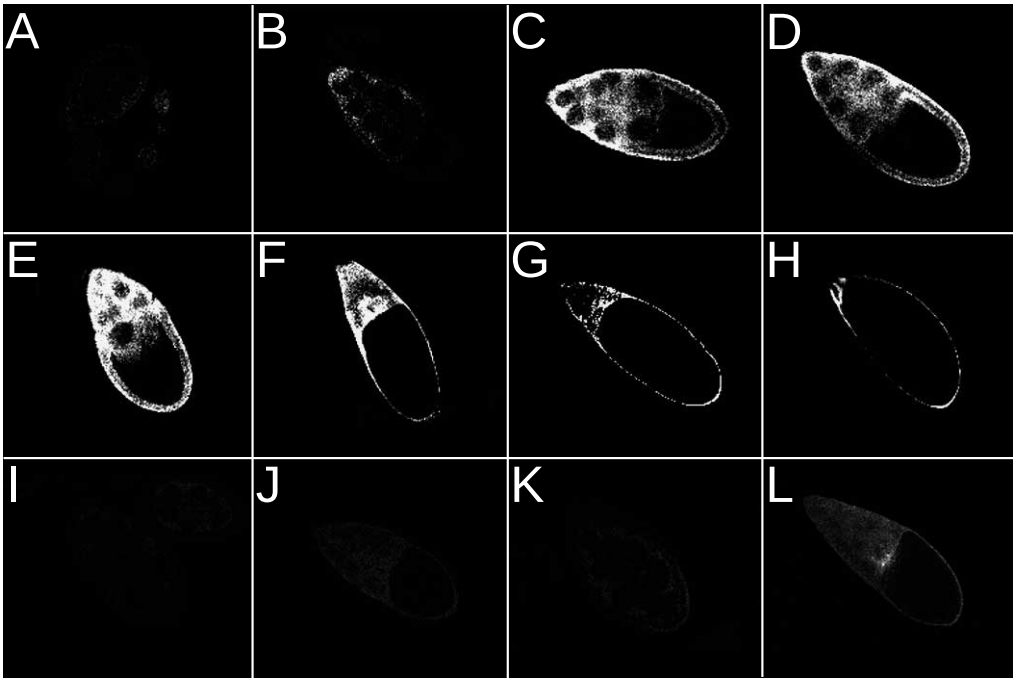

Fig. 4. Expression of Integrase protein during *Drosophila* oogenesis. Immunoflurescence analysis using confocal microscopy of *gypsy* Pol proteins using monoclonal antibody 8H10. (A) Stages 1-8 egg chambers from females carrying the *flam* mutation; only background level staining can be observed at this time of development; (B) stage 9 egg chambers of *flam* ovaries showing initial expression levels of IN protein; (C) stage 10A; (D) stage 10B; (E) stage 11; (F) stage 12; (G) stage 13; (H) stage 14; (I) stages 1-9 of egg chamber development in ovaries from strain SS females; (J) stage 10 of oogenesis in SS females; (K) stages 1-9 of oogenesis in *flam*/+ females; (L) stage 10 *flam*/+ egg chamber.

perivitelline membrane (Fig. 5C,D). These virus-like structures have a diameter of approximately 100 nm and can be seen in the perivitelline membrane during later stages of oogenesis up to stage 14 (Fig. 5E). No Env cross-reacting material or virus-like structures can be detected in egg chambers from heterozygous *flam*/+ females (Fig. 5F). These results suggest that *gypsy* viral particles are assembled in the follicle cells starting at stage 9 of oogenesis; these particles apparently bud out of the follicle cells during early stage 10 before formation of the vitelline membrane has been completed and could thus infect the oocyte during a brief developmental window of opportunity. This hypothesis is supported by the observation of virus-

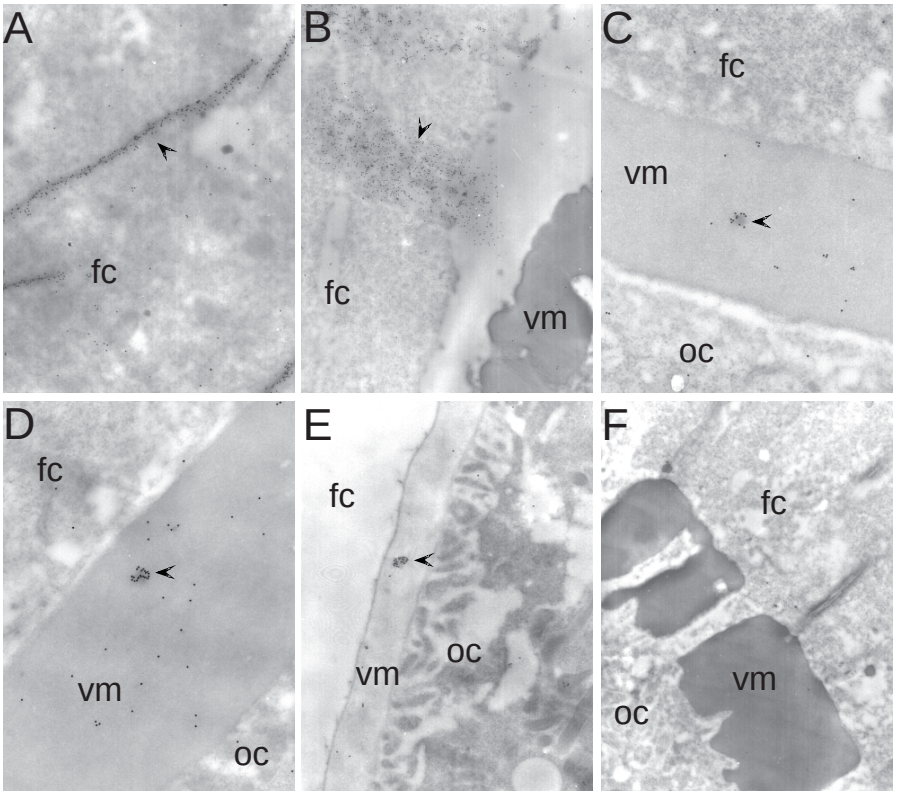

Fig. 5. *Gypsy* viral particles are present in *flamenco* egg chambers. (A) Immunoelectron micrograph of thin sections from egg chambers of *flam* females at stage 9 of oogenesis. Gold particles indicate the presence of *gypsy* Env protein in the endoplasmic reticulum (arrowhead). Magnification 31 500. (B) Immunolocalization of Env protein with 7B3 antibody using electron microscopy during stage 10A of oogenesis in *flam* females. Arrowhead indicates the presence of Env protein inside the cytoplasmic membrane. Magnification 12 500. (C) Viral particle cross-reacting with 7B3 antibody visualized by immunoelectron microscopy of sections from *flam* ovaries during stage 10B of oogenesis. The particle is trapped in the newly formed vitelline membrane. Magnification 25,000. (D) A similar 7B3-cross reacting viral particle in a stage 11 egg chamber from a *flam* female. Magnification 25 000. (E) *Gypsy* viral particle present in the vitelline membrane of a stage 14 egg chamber of a *flam* female. Magnification 20 000. (F) Immunoelectron micrograph of a stage 10 egg chamber from the SS strain probed with monoclonal antibody 7B3; neither cytoplasmic staining nor viral particles can be observed in egg chambers of this strain. Magnification 20 000. vm, vitelline membrane; fc, follicle cell; oc, oocyte.

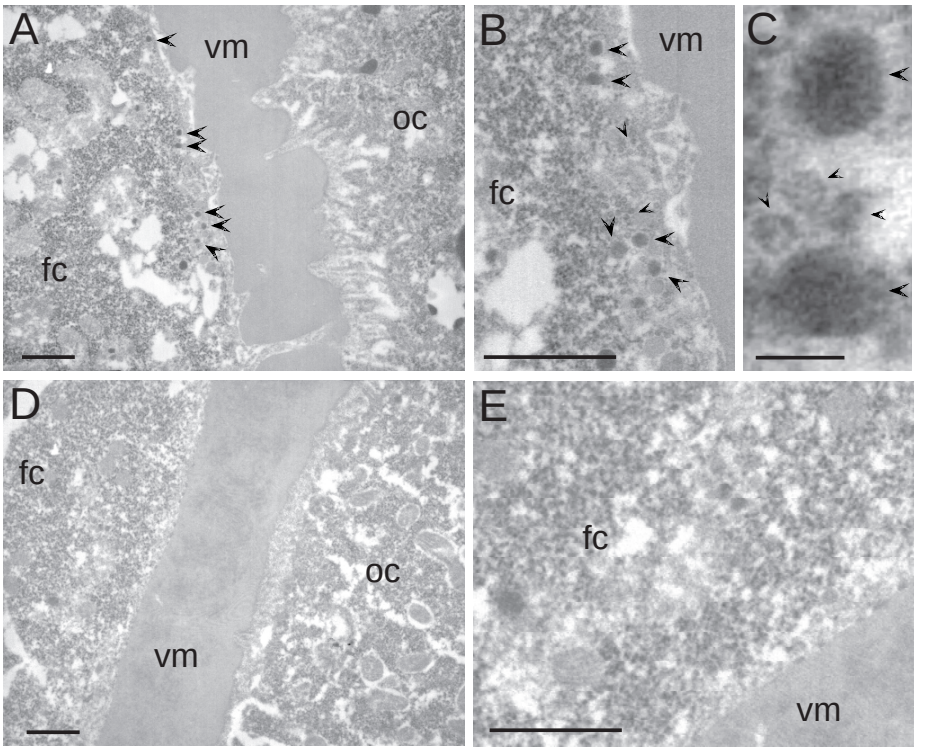

Fig. 6. Presence of putative *gypsy* core particles in follicle cells of *flam* females. (A,B) Low (10 000) and high (28 000) magnifications respectively of the interface between the apical region of a follicle cell and the oocyte from egg chambers of *flam* females. Large arrowheads indicate the presence of 100 nm putative *gypsy* core particles during the viral assembly process; small arrowheads indicate 35-40 nm particles of unknown identity. Bar in each panel corresponds to 1 μm. (C) Higher magnification (84 000) of two 100 nm particles indicated with large arrowheads; small arrowheads point to 35-40 nm particles. Bar corresponds to 100 nm. (D,E) Low (10 000) and high (28 000) magnification, respectively, of the apical region of a follicle cell from strain SS females. Bar in each panel corresponds to 1 μm. vm, vitelline membrane; fc, follicle cell; oc, oocyte.

like particles embedded in the vitelline membrane, where they might have been trapped during the formation of this structure, at later stages of oogenesis.

Gypsy viral particles assembling in the follicle cells may not be apparent in the experiments described above due to the light fixation methods employed during immunoelectron microscopy experiments. To avoid this problem, thin sections of ovaries fixed with glutaraldehyde and stained with uranyl acetate were examined by transmission electron microscopy. The results of this experiment are shown in Fig. 6. Sections of egg chambers from *flam* females during stage 10A of oogenesis contain structures resembling retroviral core particles that accumulate in the cytoplasm of the follicle cells adjacent to the basal membrane (Fig. 6A-C); these particles are approximately 100 nm in diameter, corresponding well in size with the *gypsy* virions observed by immunoelectron microscopy (Fig. 5) and those purified from extracts of ovaries from *flam* females (Song et al., 1994). Although there is no direct evidence at this time that these structures correspond to *gypsy* viruses, they are present only in *flam* egg chambers, at the same time and subcellular location as Env protein accumulates in these cells. In addition, we observe much smaller (35-40 nm) round particles with an electron-lucent core in this same part of the follicle cells. These structures are not associated with ORF3 antibody, so the identity of these small particles is uncertain and must await analysis with anti-Gag antibodies. Follicle cells from SS strain females lack both types of virus particles (Fig. 6D,E).

DISCUSSION

Mobilization of *Drosophila* retroelements, and in particular the *gypsy*-related retrovirus, in the genome of the host is a rare phenomenon. Most laboratory strains contain only a few euchromatic copies of *gypsy*, suggesting that the host has developed mechanisms to control the germ-line mobilization of this and other retroelements. The rate-limiting step of *gypsy* mobilization seems to be controlled by the product of the *flamenco* gene, since *flam* mutations result in high rates of *de novo gypsy* insertion (Mével-Ninio et al., 1989; Prud'homme et al., 1995). Mutations in *flam* display a typical maternal effect and the *flam* gene product appears to act at the level of splice site selection; in the absence of the *flam* protein, the *gypsy* full-length RNA is processed to give rise to an *env*-specific transcript in which RNA sequences located between the beginning of the Gag and Env open reading frames have been eliminated. In addition, and probably as a consequence of this splicing event, accumulation of the full-length *gypsy* transcript also increases in the ovaries of *flam* females (Pélisson et al., 1994). The question then is how expression of the *env*-specific RNA results in *gypsy* insertion into the germ line of the progeny of *flam* females.

Processing of *gypsy* Env and Pol proteins extends analogy to retroviruses

We have previously shown that the *env* transcript is expressed to give rise to a protein that is glycosylated and present in the follicle cells of late stage 10 egg chambers from *flam* females (Song et al., 1994; Pélisson et al., 1994). Results presented here extend the similarities between the putative *gypsy* Env protein and that of vertebrate retroviruses. Monoclonal antibodies raised against epitopes located in the putative surface and transmembrane proteins have been used to establish that the product of *gypsy* ORF3 is processed in a manner expected for a true retroviral Env, supporting the idea that *gypsy* can encode infectious retroviral particles, able to assemble only in the ovaries of *flam* females. In addition, other components of *gypsy* particles are also present in the follicle cells at the same time

as Env: monoclonal antibodies against a putative product of the *pol* open reading frame indicate the presence of a putative Integrase mature protein.

The presence of *gypsy*-encoded proteins, including Env, in egg chambers of *flam* females supports the idea that *gypsy* retroviral particles can be assembled in the ovaries of these flies. In fact, *gypsy* viral particles have been observed by electron microscopy in extracts from ovaries of *flam* females, and these particles are infectious as concluded from experiments in which larvae that have been fed particles give rise to progeny containing additional copies of *gypsy* in novel genomic locations (Kim et al., 1994; Song et al., 1994). These particles could not arise as a consequence of Gag and Pol expression from full-length *gypsy* RNA present in nurse cells since, a third required component, Env protein, is not made in these cells. If mobilization of *gypsy* in the progeny of *flam* females requires the presence of *gypsy* particles in the oocyte and *gypsy* is only fully expressed in the somatic follicle cells, the presence of *gypsy* viral particles in the oocyte can only be explained if particles formed in the follicle cells infect the oocyte. This type of mechanism has been previously observed in the congenital transmision of avian leukosis virus (ALV) in chickens (Di Stefano and Dougherty, 1966). ALV-infected hens produce viral particles in the albumen-secreting glands of the magnum during ovulation; viral particles are also produced by theca and follicular epithelial cells, and they accumulate in the extracellular connective tissue spaces of the oviduct and ovary. Infection of the oocyte by these particles is presumably responsible for the infection observed in the progeny of these hens (Di Stefano and Dougherty, 1966).

Parallels with vertebrate retroviruses

The process of oocyte infection by closely apposed follicle cells has many parallels with retroviral infection of polarized cells, such as mammalian endothelial cells. These cells are the essential component of the blood-brain barrier that appears to separate circulating retroviruses such as HIV from the brain, but often does not as in the case of AIDS-related dementia, which is characterized by HIV infection of brain cells. The mechanism by which this occurs is controversial, but there is considerable evidence that HIV (and other retroviruses) can infect endothelial cells. When murine retroviruses infect endothelial cells, virus assembly occurs exclusively on the basement membrane surface of these cells (Bilello et al., 1986; Pitts et al., 1988). In a related phenomenon, many investigators have noted that retroviral virion assembly processes are associated with different intracellular compartments in different cell types. HIV virus buds mostly into vacuoles in the cytoplasmic membranes of macrophages whereas it buds at the plasma membrane surface of leukocytes. The fate of the macrophage vacuolar virus is uncertain, but is reminiscent of some of the structures that we observed in follicle cells. In addition, the sheep lentivirus visna virus non-randomly buds from specific domains (specifically, near cell edges) of endothelial cells grown on a fibronectin-coated membrane (C. Zink, personal communication). If retroviruses indeed cross the blood-brain barrier into the CNS by directionally infecting and crossing endothelial cells, this is strikingly similar to our result that the *gypsy* virions assemble in only a single surface domain of the follicle cells – the surface that is closely apposed to the target cell, the oocyte.

A developmental window of opportunity for oocyte infection

The timing of infection of the *Drosophila* oocyte by follicle-cell-produced *gypsy* viral particles has to be carefully orchestrated: after stage 10 of oogenesis, the oocyte becomes completely surrounded by the thick impermeable vitelline membrane, which would presumably impose an impenetrable barrier to viral infection. *Gypsy* Env protein accumulation is low early in oogenesis and rises during stage 9, but Pol proteins are not synthesized at measurable levels before this time. Therefore, *gypsy* has a small window of opportunity to form viral particles and infect the oocyte. The observation of these particles trapped within the vitelline membrane at later stages of development is a good indication that this actually occurs. Failure to observe these particles at earlier stages, before vitelline membrane formation, is probably a consequence of low levels of *gypsy* Env expression at this time of oogenesis as well as the difficulty in visualizing the follicle cell/oocyte interphase in lightly fixed preparations for EM immunocytochemistry. Nevertheless, structures resembling viral core particles can be observed in conventionally prepared thin section of egg chambers at the appropriate developmental stage. Although we can not conclusively establish the identity of these particles, the fact that they are present in egg chambers from *flam* females but not females from strains that do not support *gypsy* mobilization suggests that they correspond to *gypsy* viruses.

A model for germ-line insertion by *gypsy*

The fate of *gypsy* particles after oocyte infection is unclear at this time. Once in the oocyte, the particles are presumably devoid of Env protein and thus not detectable by immunological methods using anti-Env antibodies. Their fate can be inferred from the pattern of ovo^D reversion events in the progeny of *flam* females arising from the infected oocyte. *Gypsy* viral particles present in the oocyte after infection and presumably transmitted to the embryo serve as substrate for reverse transcription and subsequent integration of the double-stranded DNA (Fig. 7). *Gypsy* insertion seems to be limited to germ-line cells during embryonic development of the progeny based on the following. Somatic insertion events during embryogenesis into X-linked genes with visible phenotypes such as *white*, *yellow*, *forked*, etc., would give rise to large patches of mutant tissue that could be detected in the adult; since this is not observed, we infer that early integration into the chromosomes of somatic nuclei or cells might not take place in the embryo. On the contrary, since this is a negative result, low levels of somatic transposition might be difficult to detect.

The observed transposition of *gypsy* in the germ line can be explained if *gypsy* particles accumulate in the cytoplasm at the posterior end of the egg before cellularization occurs. When the pole cells form at stage 7 of embryogenesis, *gypsy* particles could accumulate in the cytoplasm of these cells when membranes are formed and cellularization takes place. This process is diagrammed in Fig. 7C,D, where the pole cells are depicted after membranes have formed, surrounding the cytoplasm previously located in the oocyte and engulfing

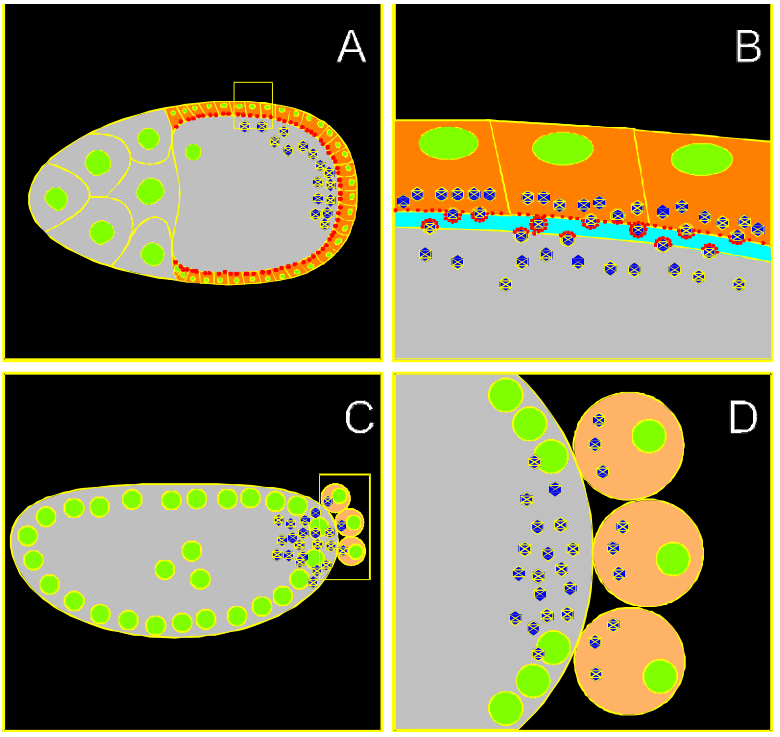

Fig. 7. A model for *flam*-induced *gypsy* mobilization. (A,C) Diagrams of a stage 10 egg chamber and a preblastoderm embryo. (B,D) Magnifications of selected areas demarcated by rectangles in A and C. *Gypsy* viral particles (dark blue with diagonal cross) assemble in the follicle cells and traverse the perivitelline space (light blue) before vitelline membrane formation. The viral particles infect the oocyte and the core particles lacking Env (red dots) are transported to the posterior end. In the embryo, posteriorly localized core particles become included in the pole cell cytoplasm upon cellularization (D). The pole cells shown in C and D have already cellularized, forming membranes that separate their cytoplasm from that of the oocyte. Before this occurs, the pole cells and oocyte have a common cytoplasm and particles present in the oocyte can become engulfed into the pole cell cytoplasm when cellularization takes place. These particles contain *gypsy* full-length RNA and reverse transcriptase to sustain the synthesis of double-stranded DNA. This DNA will serve as a substrate for integrase in the process of *gypsy* insertion into the genome of germ-line cells.

gypsy particles present in the region. The timing of *gypsy* integration into the chromosomes of germ-line cells might be inferred from the number of progeny arising from ovo^D revertant females. If integration takes place in the pole cells early during embryogenesis, ovo^D revertants should have a normal number of progeny, and this number should decrease as the time of the integration event shifts to later stages of development. ovo^D revertants arising from *flam* females give rise to between 20% and 100% of the normal number of progeny, suggesting that *gypsy* integrates into pole cell DNA early in embryonic development.

The mechanism for preferential integration of *gypsy* into pole cells versus the rest of the somatic cells of the embryo is unclear. After infection of the oocyte, *gypsy* particles would be devoid of Env protein and thus unable to infect other cells (Fig. 7). Mobilization of *gypsy* takes place in the *flam*/+ progeny of *flam* females, suggesting that the *flam* mutation, and therefore synthesis of Env protein, is not required in the embryo for *gypsy* integration provided the mother carried the mutation. This suggests that cell invasion by *gypsy* does not require more than one round of infection/replication and may take place in the preblastoderm stages before cellularization (Fig. 7). The specificity of germ versus somatic cells might then simply rely on the posterior localization of *gypsy* particles within the developing embryo. We propose that, after infection of the oocyte, *gypsy* particles are transported to the posterior end, taking advantage of processes in use at this time to set up the anterior-posterior polarity of the embryo. Localization of these particles to the posterior region of the embryo, together with posterior determinants present in pole granules, would result in their localization in the cytoplasm of pole cells that will form the germ line (Fig. 7). An alternative possibility to explain the germ-line specificity is that infectious viral particles remain in the perivitelline space through cellularization and infect the pole cells or their descendants at this or subsequent stages of development, due to the presence of specific receptors in the membrane of these cells. Experiments now in progress using anti-Gag antibodies to detect *gypsy* core particles devoid of Env will allow us to test these models.

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