Life Cycle of an Endogenous Retrovirus, ZAM, in Drosophila melanogaster

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ZAM is an env-containing member of the gypsy family of retrotransposons that represents a possible retrovirus of invertebrates. In this paper, we traced ZAM mobilization to get information about a potential path a retroelement may take to reach the germ line of its host. In situ hybridization on whole-mount tissues and immunocytochemistry analyses with antibodies raised against ZAM Gag and Env proteins have shown that all components necessary to assemble ZAM viral particles, i.e., ZAM full-length RNAs and Gag and Env polypeptides, are coexpressed in a small set of follicle cells surrounding the oocyte. By electron microscopy, we have shown that ZAM viral particles are indeed detected in this somatic lineage of cells, which they leave and enter the closely apposed oocyte. Our data provide evidence that the vesicular traffic and yolk granules in the process of vitellogenesis play an important role in ZAM transfer to the oocyte. Our data support the possibility that vitellogenin transfer to the oocyte may help a retroelement pass to the germ line with no need of its envelope product.

ZAM is a 8.4-kb retroelement that resides within the genome of Drosophila melanogaster (11). On the basis of sequence similarity and gene organization, ZAM is a member of a group of retrotransposons that bears a striking resemblance to the vertebrate retroviruses. These elements are flanked by long terminal repeats (LTRs) that direct the transcription of full-length RNAs representing potential templates for reverse transcription during mobilization. The LTRs flank three open reading frames (ORFs) analogous in position and coding potential to the retroviral gag, pol, and env genes (Fig. 1). Among the diverse classes of eukaryotic retrotransposons, the presence of a third env-like ORF (ORF3) is unique to ZAM and a small group of other members of this family, including gypsy, 297, 17.6, Idefix, and nomad in D. melanogaster (3, 8, 14, 19, 26), tom in Drosophila ananassae (25), Osvaldo in Drosophila buzzatii (15), TED in the lipedopteran Trichoplusia ni (5), and Yoyo in the medfly Ceratitis capitata (28). An envelope protein expressed in vivo has been identified for only three of these elements (gypsy, tom, and TED) (16, 21, 24, 25), and only one of them, gypsy, has been shown to date to have infectious properties (9, 22). Although retroviral Env proteins are known to be involved in viral infectivity through host cell receptor recognition and fusion of viral and cellular membranes, the role of the Env glycoproteins encoded by these elements is still unclear since no budding has ever been visualized for any of them.

ZAM was first identified as a spontaneous insertion at the white locus, giving rise to the wIR6RevI allele in a line of D. melanogaster subsequently called RevI (11). This mutation occurred in the course of a massive amplification of ZAM elements in this line due to their mobilization, which remains active in this stock of flies (3). The existence of RevI and its parental line, wIR6, which displays a low copy number of stable ZAM elements, offers a good genetic system where the control of ZAM mobilization and its relationship with its host genome may be studied. Indeed, we previously reported that ZAM transcription is active in RevI and inactive in wIR6. Two kinds of transcripts similar to mRNAs synthesized from a vertebrate retrovirus involved in a replication cycle were identified in RevI. One corresponds to a full-length genomic RNA, and the other corresponds to a subgenomic transcript of the ORF3 gene able to encode a protein which displays all the features of retroviral envelope proteins. Due to the presence of these transcripts in the course of ZAM mobilization, an important issue is to know whether its entire replication cycle is identical to that of insect retroviruses and involves an extracellular step before ZAM integration in the genome.

We initially reported that ZAM is mobilized through a reverse transcription process occurring in the germ line of flies (11). In this paper, we searched for tissues where ZAM is transcribed, translated, and potentially assembled in viral particles.

We report that ZAM RNAs are detected in a very specific somatic lineage of cells located around the oocyte in the ovaries of the RevI line. Using polyclonal antisera raised against bacterial ORF1- and ORF3-encoded ZAM fusion proteins, we show that both proteins are coexpressed with the full-length ZAM RNAs in these follicle cells surrounding the oocyte. Furthermore, we provide evidence that particles of ZAM are formed in these follicle cells and pass to the oocyte via the vitelline granule traffic with no apparent need for its Env protein.

MATERIALS AND METHODS

Fly stocks. The wIR6 and RevI strains (low copy number and high copy number of ZAM, respectively) are from the collection of the Institut National de la Sante et de la Recherche Médicale U384.

In situ hybridization. Embryos at different stages were collected on grape juice agar plates, and fly stocks were maintained on cornmeal-glucose-yeast media at 20°C.

Ovaries and testes were dissected in 1× phosphate-buffered saline (PBS). Dissected ovaries, testes, and embryos were fixed in heptane-saturated 5% paraformaldehyde-0.1 M HEPES (pH 6.9)-2 mM MgSO4-1 mM EGTA for 20 min. Ovaries were rinsed with PBTr (PBS, 0.1% Tween 20) and 0.1 mg of heparin, 0.1 mg of salmon sperm DNA, and 0.1 mg of tRNA/ml, 0.1% Tween 20), in a 1/1 mixture of hybridization solution (55.5% formamide, 0.25× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5 mg of heparin, 0.1 mg of salmon sperm DNA, and 0.1 mg of tRNA/ml, 0.1% Tween 20), in a 1/1 mixture of hybridization solution.
solution and PBT at 55°C for 30 min each, and in PBT at room temperature (two washes for 20 min each). The hybridized probe was detected using the Genus Kit (Boehringer).

DNA constructs, protein purification, and generation of polyclonal antibodies. The ZAM gag ORF was amplified with the Expand long-template PCR system (Boehringer) on RevI genomic DNA with oligonucleotide 1 (5'-GAGATCTCA AACAACTCCTCCCTGTTA-3'; positions 1819 to 1839) and oligonucleotide 2 (5'-GAGATCTCCTATATGTGGATACCC-3'; positions 2805 to 2823) (Fig. 1A) with dGt114 nucleotides 1 and 2 display at their 5' ends BglII and EcoRI restriction sites, respectively. The gag PCR product was inserted into the pGEX4T-2 vector (Pharmacia Biotech) for glutathione S-transferase (GST)-Gag fusion protein production in the bacterial BL26 strain. The GST-Gag fusion protein was purified by chromatography with glutathione immobilized on cross-linked 4% beaded agarose (Sigma). In order to test the anti-Gag polyclonal antibody, the ZAM gag PCR product was subcloned into the pRSETB vector for His-Gag fusion protein production (see Results).

A 0.7-kb BglII-DraI DNA fragment (Fig. 1) encoding the N-terminal part of ZAM Env protein was subcloned from the BH clone (11) into the pRSETB vector for histidine fusion protein production in the bacterial BL21 strain according to the manufacturer protocol (Invitrogen). The histidine-Env fusion protein was purified according to the manufacturer protocol for chromatography on a nickel affinity resin (Invitrogen).

The purified GST-Gag and histidine-EnvN fusion proteins were used for generation of polyclonal antibodies in rabbits and rats, respectively (Eurogenetec).

Whole-mount immunocytochemistry. Ovaries were dissected in cold 1× PBS and fixed in 5% formaldehyde–1× PBS–50 mM EGTA–25% (vol/vol) heptane for 20 min. They were treated in methanol andcopiously rinsed in 1× PBS. Immunodetections were performed with AbC Vectastain kit (Vector Laboratories) using anti-rat or Texas red-conjugated antirabbit antibodies at dilutions of 1/200 and 1/600, respectively (Molecular Probes). Ovaries were embedded in Mowiol 4.88 (Calbiochem) prepared as described by the manufacturer at pH 8.5. Whole-mount ovaries were scanned on the Leica confocal microscope. Optical sections were 2 μm thick.

Ultrastructural studies. For ultrastructural studies 2- to 3-day-old flies were dissected in PBS, and the ovaries were quickly fixed for 2 h in ice-cold 5% glutaraldehyde–4% formaldehyde in 0.1 M cacodylate buffer at pH 7.2. Individual ovarian follicles were separated from the ovaries while in the fixative, fixed for 2 h in 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2 and rinsed again in the same buffer. Ovarian follicles were then dehydrated in a graded series of alcohols, passed through propylene oxide, and finally polymerized in epoxy resin for 3 days at 60°C.

For immunocytochemical detection of viral antigens, ovarian follicles were fixed for 2 h in 1% glutaraldehyde–4% formaldehyde in 0.1 M buffer at pH 7.2. After dehydration in alcohols, ovarian follicles were embedded in Unicryl resin and allowed to polymerize under a UV lamp at 4°C for 3 days. Sections were obtained with an LKB ultramicrotome and mounted on uncoated nickel grids. To detect the presence of viral antigens by gold immunocytochemistry, a number of ovarian follicles were dissected and fixed in formaldehyde and then incubated for 3 h in primary mouse or rabbit (pAbGag) or anti (pAbEnv) antibodies diluted 1:500 in PBS. Ovarian follicles were then thoroughly rinsed in PBS and incubated for an additional hour at room temperature with either gold-tagged secondary goat anti-mouse immunoglobulin G (20 nM) or anti-rat (10 nM) antibodies (NCI) diluted 1:200 in PBS. Grids were conventionally stained with uranyl acetate and lead citrate and eventually observed in a Jeol EM transmission electron microscope.

RESULTS

ZAM is transcribed in the somatic follicle cells surrounding the oocyte. The pattern of spatial expression of ZAM was determined by in situ hybridization with an antisense-specific riboprobe of the ZAM env gene labeled with digoxigenin. (Fig. 1 and 2c; see Materials and Methods). This probe potentially recognizes the two ZAM transcripts identified through Northern blot analyses, i.e., the full-length 8.6-kb transcript and the 1.7-kb subgenomic transcript of the env gene (11). A ZAM-specific expression pattern was observed with this probe in the RevI strain, where ZAM mobilization is active. These transcripts were detected in the gonads and in the central nervous systems (CNS) of late embryos (>10 h). No hybridization was detected in early embryos (<8 h). The signal observed in the CNS was detected in almost all the embryos, while the prominent expression in gonads occurred in about 50% of the embryos, suggesting that this signal could be restricted to one sex (Fig. 2B). When a sense strand-specific probe for the env gene of ZAM was used to probe RevI embryos, no signal was observed in the gonads of the embryos (data not shown).

In situ hybridization of late embryos of the wiso strain, in which ZAM elements are stable, did not give any signal with the antisense-specific riboprobe of the ZAM env gene although a very faint hybridization may be detected in gonads after a very low exposure time (Fig. 2E). This result corroborates those found by Northern blotting analysis (11), indicating that ZAM mobilization is accompanied by elevated RNA levels in RevI.

Since ZAM mobilization is known to occur in the germ line of flies, we then investigated ZAM transcription in the genital apparatus of adult flies. Testes and ovaries were dissected from wiso and RevI strains (see Materials and Methods) and subjected to in situ hybridization experiments with the riboprobes described in Fig. 1. ZAM RNAs were visualized in RevI ovaries (Fig. 2C and D). Whatever the probe used, no transcript was detected in RevI or wiso testes or in wiso ovaries (Fig. 2F).

In insects, ovaries are composed of developing egg chambers arranged in tubular structures called ovarioles (Fig. 2A). The Drosophila ovary consists of 15 to 18 ovarioles. Each ovariole contains a series of egg chambers at progressively more advanced stages of oogenesis (10, 23). At the tip of each ovariole, the stem cells of the germ line and the follicle cell precursors reside in the gerarium. During oogenesis, the germ line stem cells and follicle cells go through a defined set of division cycles and become organized into egg chambers, which progressively leave the gerarium and continue developing as they move posteriorly within the ovariole. The mature egg chamber consists of the oocyte and 15 nurse cells, which are both surrounded by a monolayer of somatic follicle cells (13, 23).

During oogenesis, ZAM transcription occurs very early in the gerarium of each ovariole and then is detected in the follicular cells of each egg chamber. However, ZAM RNAs are not present in all the follicle cells but are restricted to a patch of follicle cells located at the posterior side of the oocyte. ZAM expression persists until late stages of oogenesis (Fig. 2C and D). Similar experiments performed on RevI ovaries with the sense riboprobe did not reveal any hybridization signal (Fig. 2G).

Since the env riboprobe used to detect these transcripts did not allow discrimination between the presence of the full-length transcript and the presence of the ORF3 subgenomic
FIG. 2. In situ hybridization to whole-mount embryos and ovaries to visualize the distribution of ZAM transcripts. (A) Schematic representation of an adult ovariole. The ovariole is composed here of the germarium (early stages of oogenesis) and later of two follicles in stages 9 and 10. The germ line cell nuclei (nurse cells and oocyte nuclei) are in grey, and the somatic cell nuclei (follicle cell nuclei) are in black. (B to F) In situ hybridizations of ZAM RNAs with the antisense env riboprobe (Fig. 1); (G) similar experiment with a sense env riboprobe. (B) Late embryos (up to 10 h at 20°C) of the RevI strain. Arrowheads, hybridization signals located in gonads. (C) Ovariole of RevI female. Arrowheads, hybridization signals located in the somatic follicular cells at the posterior part of each follicle and in the germarium. (D) Higher magnification of RevI follicles (stage 10). Strong hybridization signals are observed (arrowheads) in the posterior follicular cells. (E) Late embryo of the wIR6 strain. After a long time of revelation, a leaky signal is observed in the gonads (arrowheads). (F) Ovariole of the wIR6 strain. No signal is detected in the follicular cells and the germarium. (G) Negative control of RevI follicle hybridized with the sense env riboprobe. In all panels, the anterior part of an embryo or ovariole is at the left margin.
transcript, a specific ZAM riboprobe of the pol gene (Fig. 1) was used for additional in situ hybridizations. The same pattern of expression as the one described above with the ORF3 probe was observed, indicating that full-length ZAM RNAs are present in the follicle cells (data not shown).

We then addressed the question whether proteins encoded by ZAM ORFs could be detected in the cells where ZAM transcripts have been visualized.

The ORF1-encoded ZAM polypeptide is present in cells where ZAM transcription is occurring. Retroviral Gag proteins are synthesized from full-length RNAs as Gag and Gag-Pol fusion polyproteins and are assembled into virus-like particles in cells where these RNAs are detected. Gag structural polyproteins constitute the core of the viral particle. In order to know whether ZAM Gag proteins are synthesized in tissues where full-length transcripts have been detected, we performed immunocytochemistry experiments.

A purified bacterial GST-Gag fusion protein encompassing the whole length of ZAM Gag was prepared and injected into rabbits (see Materials and Methods). A polyclonal antibody denoted pAbGag, which potentially recognizes the pGag region (Fig. 1) of Gag, was obtained. We verified that the pAbGag antibody is raised against the Gag protein and not exclusively against the GST peptide present in the fusion protein. For that purpose, the gag ORF was subcloned into the Tag-histidine pRSETB vector and a histidine-Gag fusion protein was expressed. From Western blotting experiments, we found that pAbGag clearly reacts with the histidine-Gag fusion protein (data not shown).

We then examined the pattern of spatial and developmental accumulation of Gag products during different stages of Drosophila development where full-length ZAM RNAs had been previously identified. Using pAbGag for immunocytochemical experiments, we detected ZAM Gag proteins in all egg chambers of the RevI strain with the same distribution as ZAM RNAs (Fig. 3A and B). A strong immunostaining was revealed

FIG. 3. Expression of Gag and Env proteins during Drosophila oogenesis. Shown is the immunolocalization of Gag and Env proteins using polyclonal pAbGag and pAbEnv antibodies, respectively, in ovaries of RevI and w IR6 females. (A to C) Gag of ZAM revealed with the pAbGag antibody in a RevI ovariole. (A) Strong immunostaining is detected in each follicle in a few somatic follicle cells which surround the oocyte and in all follicles. (B) Higher magnification of the posterior part of an early stage 10 follicle. Staining is indicated by arrowheads. (C) Stage 10 follicle from a w IR6 female. No Gag proteins are detected by the pAbGag antibody. (D to F) ZAM Env revealed with the pAbEnv antibody in a RevI ovariole. (D) High level of staining can be observed in a few somatic follicle cells at the posterior part of early stage 10 follicles (arrow). (E) High magnification of the posterior region of the follicle. (F) Stage 10 follicle from a w IR6 female. No Env proteins are detected by the pAbEnv antibody.
in a few follicle cells located at the posterior part of each egg chamber (Fig. 3A). At stage 10, Gag immunostaining strongly underlined an area located at the frontier of the follicle cells and the oocyte. This signal tended to extend around the oocyte from stage 10 (Fig. 3B).

No immunostaining was detected in RevI embryos or larvae or when controls were performed using pAbGag antibody on w^{Ros} ovaries (Fig. 3C). Preimmune serum on RevI ovaries did not produce any immunostaining (data not shown).

Translation of ZAM ORF3 is restricted to a defined developmental window of oogenesis. ZAM encodes a subgenomic mRNA of 1.7 kb whose sequence predicts a protein with structural motifs typical of retroviral Env proteins, i.e., a signal peptide, a potential transmembrane domain, putative N-glycosylation sites, and cysteine residues. In order to determine whether this predicted Env protein is indeed synthesized in tissues where ZAM RNAs have been detected, immunocytochemistry experiments on late embryos, larvae, and dissected ovaries from RevI female strains were performed.

To this end, a bacterial histidine-ORF3 peptide fusion protein, in which the coding sequence for the ORF3 peptide was underlined an area close to the apical plasma membrane (Fig. 5A and B). Some viral particles were also detected within the oocyte at stages 8 to 10 of oogenesis. At these stages, the viral particles detected within the follicle cells have been seen in contact with the membrane enclosing the secretory granules containing the vitelline membrane precursors or even bound to the granule content itself (Fig. 6C). In the w^{Ros} strain, no particles are associated with the secretory granules (Fig. 5C, inset).

Although the mobilization of ZAM occurs within the germ line of RevI, no virus budding along the apical plasma membranes of the follicle cells was observed in this study. In this context, it is interesting to note that the vitelline membrane precursors synthesized within the follicle cells are released from this somatic lineage and pass to the extracellular region bordering the oocyte. Thus, the viral particles may benefit from their association with the vitelline membrane precursors to sort out this somatic lineage. Viral particles detected in more developmentally advanced ovarian follicles are stockpiled along the apical follicle cell plasma membrane, as if extracellular release of residual viral particles would have indeed been impeded by completion of the vitelline membrane (Fig. 6B).
FIG. 4. ZAM gag and env genes are coexpressed in the follicle cells of RevI follicles. Double staining of RevI follicles with Gag antibody (red) and Env antibody (green) in early stage 10 of oogenesis (upper panels) and in late stage 10 (lower panels). Bars: upper panels, 30 μm; lower panels, 10 μm. oo, oocyte; fc, follicle cells.
Gold immunocytochemical experiments localize Gag and Env proteins of ZAM at sites where particles accumulate. To ascertain that the ring-shaped particles observed in the posterior-most follicle cells of the RevI ovaries are indeed due to ZAM expression, a number of ovarian follicles at stages 9 and 10 were treated for the immunocytochemical detection of ZAM proteins. When tested with anti-Gag antibodies, the most heavily labeled sites of the follicular epithelium appeared to be those cells that face the posterior pole of the oocyte (Fig. 7A). Within the follicle cell cytoplasm gold label accumulated along the apical end, even though the basolateral borders were also labeled to some extent (Fig. 7A and B). Along the apical border, gold particles were preferentially associated with the vitelline membrane precursors or, extracellularly, with the deposited vitelline membrane.

In the cortical ooplasm, gold label appeared dispersed among endocytic vesicles (Fig. 7C). Yolk granules were also labeled, but the gold particles over these organelles occurred more frequently inside the so-called superficial layer than within the enclosed main body (Fig. 7D). In ovarian follicles at a more advanced developmental stage of oogenesis than stage 10, the label tended to gradually disappear both from the follicle cell cytoplasm and the oocyte (data not shown).

When tested with anti-Env antibodies, ovarian follicles appeared labeled over both the follicle cell cytoplasm and the oocyte (Fig. 8A), with gold particles occurring along the apical follicle cell membrane (Fig. 8B) and the cortical ooplasm among the endocytic vesicles (Fig. 8C). Label appeared to persist along the oocyte plasma membrane even in ovarian follicles with a complete vitelline membrane and no endocytic uptake (Fig. 8D).

As a general rule, the gold labeling due to anti-Env antibodies is low and does not spatially coincide with viral particles, indicating that the 45- to 50-nm particles may correspond to ZAM particles devoid of an envelope. Although the ZAM Env protein is associated with the plasma membrane, as expected
for a functional Env protein, these results suggest that the Gag-Env interaction may not have an obligatory role for the cell-to-cell transmission of ZAM.

**DISCUSSION**

The present work reports data about the mobilization of retroviral particles produced in a somatic lineage and passing to the germ line. Analysis of the cell-to-cell transmission of the ZAM retroelement of *Drosophila* permits us to propose a possible mechanism for such a mobilization.

The mobilization of ZAM correlates with the production of all components necessary to assemble virus-like particles in the follicle cells. A previous study had reported that ZAM displays all the structural features of a vertebrate retrovirus (11). However, this first study failed to determine whether the predicted products of ZAM were indeed synthesized in the course of its mobilization. This was achieved in this study.

Expression of retroviruses necessitates transcription of a full-length RNA and synthesis of retrovirally encoded proteins Gag, Pol, and Env. All these components encoded by ZAM have been detected in the ovaries of a strain where ZAM mobilization is known to be high, the RevI strain, and are absent in the w<sup>IR6</sup> line, in which ZAM mobilization does not occur. Full-length ZAM transcripts have been detected in a group of cells of somatic origin that are the follicle cells surrounding the posterior part of each oocyte.

Polyclonal antibodies raised against the putative full-length Gag protein recognized a ZAM Gag product in adult ovaries in a distribution pattern similar to that of ZAM RNAs. Gag was detected in each follicle starting from early stages of oogenesis. At stages 9 and 10A, the antibody revealed Gag proteins at the

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**FIG. 6.** Cytochemical detection of viral particles on vitelline membrane precursors and in yolk granules of the RevI strain. (A) A vitelline membrane precursor (pVm) along the apical end of a posterior follicle cell (fc) from a stage 9 ovarian follicle. Note the presence of numerous viral particles (arrowheads) around the granule periphery. Bar, 0.25 μm. Vm, vitelline membrane. (B) The apical end of a posterior follicle cell from a stage 11 ovarian follicle. Note the presence of numerous viral particles (arrowheads) along the margin of the vitelline membrane. Bar, 0.2 μm. (C) A forming yolk granule (y) from the cortical oooplasm of a stage 9 ovarian follicle of RevI following a 1-h exposure to HRP. Note that viral particles (arrowheads) are present along the superficial layer underneath the limiting membrane. A peroxidase-labeled endocytic vesicle is also visible along the membrane (arrow). oo, oocyte. Bar, 0.5 μm. (D) A forming yolk granule from a stage 9 RevI ovarian follicle fixed for 4 h with zinc osmium iodide (OZI). Note the presence of several viral particles (arrowheads) along the superficial layer among several electron-dense spots of OZI precipitates. Bar, 0.1 μm.
border between the oocyte and the follicle cells, in addition to their location in the follicle cells. Later, in stage 10B, Gag proteins were present around the oocyte while they were then absent from the follicle cells. These data are consistent with movement of Gag-containing particles between the follicle cells and the oocyte. Antibodies raised against the ZAM Env led us to visualize the presence of such a protein in a specific group of follicle cells at the posterior part of the oocyte. Although ZAM RNA and Gag proteins have been visualized in these cells, the Env pattern of expression is detected in a more restrictive pattern of development since Env proteins are only present at stages 9 and 10 of oogenesis and are absent in earlier stages.

FIG. 7. Immunocytochemical detection of Gag viral antigens. (A) The follicle cell-oocyte border from a stage 9 RevI ovarian follicle tested with anti-Gag antibody. fc, posterior follicle cell; N, follicle cell nucleus; oo, oocyte; Vm, vitelline membrane. Bar, 4 μm. (B) Enlargement of panel A to show numerous 20-nm gold grains of the secondary antibody along the apical end of the follicle cell. Bar, 1 μm. (C) Portion of the cortical ooplasm from a stage 10 RevI ovarian follicle showing gold grains (arrowheads) due to anti-Gag antibody along the oolemma. Bar, 0.5 μm. (D) A forming yolk granule (y) from a stage 9 RevI ovarian follicle. Note the presence of gold grains due to anti-Gag antibody (arrowheads) over the superficial layer among viral particles (arrows). Bar, 0.4 μm.
Virus-like particles of ZAM may benefit from exocytic and endocytic exchanges to pass from the follicle cells to the germ line. Previous experiments had indicated that novel ZAM insertions frequently occur within the germ line of RevI (11). Owing to the fact that all the components necessary for ZAM’s mobilization had been detected in a somatic lineage and that movement of Gag-containing particles had been suggested by our immunocytochemical approach, we then searched for a potential pathway leading ZAM to the oocyte. Through an ultrastructural study, ring-shaped or ellipsoidal viral particles of about 45 nm in mean diameter were detected in RevI. These particles are similar to defective human immunodeficiency virus particles that exhibit an electron-dense ring corresponding to a Gag protein not yet cleaved to yield the mature viral form (7). Several lines of evidence strongly argue that these particles correspond to ZAM particles: (i) they are detected within cells where immunostaining and confocal analysis with anti-Gag and anti-Env antibodies have revealed the presence of ZAM products, i.e., the follicle cells facing the posterior pole of the ovarian follicle; (ii) they are absent in w^Rev^, where no ZAM mobilization has been observed; (iii) immunogold cytochemistry with antibody pAbGag confirms this staining pattern by showing that the labeling of the follicular epithelium is primarily due to the ZAM product lying close to the vitelline membrane precursors.

This structural analysis brought three pieces of information that help to trace ZAM mobilization. First, the particles occur in close association with the vitelline membrane of the posterior-most follicle cells. These data indicate that the particles will be able to sort out the follicle cells when these vesicles are secreted. Second, ZAM particles are also detected within the cortical ooplasm, indicating that ZAM particles have been able to pass from the follicle cells to the oocyte. In the oocyte, the particles display a very specific distribution. Indeed, almost all of them are embedded within the superficial layer of the yolk granules along the cortical ooplasm. Third, as for ZAM Gag, Env is observed within the follicle cells specifically along the apical follicle cell membrane. However, no budding within the extracellular compartment between the follicle cells and the oocyte has ever been detected.

These overall data support the following pathway by which ZAM particles enter the RevI germ line. The initial step is to form and accumulate ZAM particles in the follicle cells. At early stage 10 of oogenesis, these particles are secreted along the apical end of the follicle cells in close association with the vitelline membrane. Gold grains are dispersed over the vitelline membrane (Vm) of a stage 10 RevI ovarian follicle exposed to anti-Env antibody. Arrowhead, gold-labeled coated vesicle. oo, oocyte. Bar, 0.5 μm. (B) The apical end of a posterior follicle cell from a stage 9 RevI ovarian follicle showing several gold grains (arrowheads) along the plasma membrane. Bar, 0.4 μm. (C) The posterior-most cortical ooplasm from a stage 9 RevI ovarian follicle tested with anti-Env antibody. Arrowhead, gold-labeled coated vesicle. oo, oocyte. Bar, 0.4 μm. (D) Portion of a stage 11 RevI ovarian follicle showing the vitelline membrane and the underneath oolemma. Vitellogenic uptake has ceased by this developmental stage in D. melanogaster, and yet gold grains due to the anti-Env antibody are still seen bound along the microvilli of the oolemma. Bar, 0.6 μm.

FIG. 8. Immunocytochemical detection of Env viral antigens. (A) The follicle cell (fc)-oocyte border from a stage 10 RevI ovarian follicle exposed to anti-Env antibody. Gold grains are dispersed over the vitelline membrane (Vm). y, yolk granule. Bar, 0.5 μm. (B) The apical end of a posterior follicle cell from a stage 9 RevI ovarian follicle showing several gold grains (arrowheads) along the plasma membrane. Bar, 0.4 μm. (C) The posterior-most cortical ooplasm from a stage 9 RevI ovarian follicle tested with anti-Env antibody. Arrowhead, gold-labeled coated vesicle. oo, oocyte. Bar, 0.4 μm. (D) Portion of a stage 11 RevI ovarian follicle showing the vitelline membrane and the underneath oolemma. Vitellogenic uptake has ceased by this developmental stage in D. melanogaster, and yet gold grains due to the anti-Env antibody are still seen bound along the microvilli of the oolemma. Bar, 0.6 μm.
vitelline membrane precursors. In more developmentally advanced ovarian follicles, extracellular release of residual particles is then impeded by completion of the vitelline membrane leading to viral particles stockpiled along the apical follicle cell plasma membrane. Once released into the follicle cell-ooocyte interface, they are transferred to the oocyte and eventually conveyed to the yolk granules, where most of them are detected.

Surprisingly, the scenario deduced from our data supports the idea that ZAM may not need its envelope for an extracellular transmission. It has already been reported that retroviruses may not require their Env proteins for budding to take place. As an example, in polarized cells, the human immunodeficiency virus type 1 Gag protein has been found to direct budding from cell membranes with no necessity for the Env glycoproteins (2). The retroviral Gag proteins play a part in the incorporation of Env into the viral particle, but they also have the capacity for packaging foreign glycoproteins (4, 27). In that context, it is interesting to suggest that Gag proteins of ZAM could recognize the vitellogenin proteins as foreign glycosylated proteins and benefit from their release out of the post-follicle cells to sort out this somatic lineage.

This potential way for a retroelement to pass from one cell to another may explain the results obtained with retroviruses expressed in a somatic lineage close to the germ line or other retroelements from insects such as gypsy. Indeed, in a genetic context permissive for gypsy mobilization, particles containing gypsy RNA have been described as preferentially clustered in the anterior follicle cells. When these cells were tested with anti-Env antibodies, gold labeling appeared almost exclusively associated with the plasma membrane from the follicle cells could be observed (12). In addition, recent data from a genetic approach have clearly demonstrated that invasion of the female germ line by gypsy retroviruses may occur in an Env-independent manner (1). Although no direct evidence was presented, the authors proposed that nonenveloped particles might enter the oocyte by endocytosis as a cytochemical tracer. One can predict that the vitellogenic traffic is potentially involved in gypsy mobilization, as suggested for ZAM from our observations.

What is then the function of the env genes of both these elements? Song et al. (21) reported that at least some gypsy elements can be enveloped and display infectious properties. Research into the role of ZAM Env and the formation of enveloped particles will certainly be the next step in understanding the ZAM life cycle. Indeed, if ZAM particles are “homed” by their Gag proteins to regions of the plasma membrane where the Env glycoproteins of ZAM reside, they may have been undetected in our experiments.

When ZAM is in the oocyte, the next step in the ZAM cycle is for it to reach the oocyte nucleus. Although this part of the ZAM cycle remains to be elucidated, the data reported in this paper bring the interesting observation that ZAM Gag may enter the nuclei of the follicle cells. Indeed, gold cytochemistry performed with pAbGag detected a nuclear staining in the follicle cells. These data could indicate that the ZAM Gag structural protein displays a specific motif responsible for directing the protein into the nuclei as already reported for foamy viruses (20). Is such a motif responsible for the entry of ZAM into the nucleus of the oocyte? Future experiments will have to clarify the pathway of ZAM to the oocyte nucleus.

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