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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 lepidopteran *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 $w^{IR6RevI}$ 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, w^{IR6}, 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 w^{IR6}. 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 infectious 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 w^{IR6} 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 4% paraformaldehyde–0.1 M HEPES (pH 6.9)–2 mM $MgSO_4$–1 mM EGTA for 20 min. Ovaries were rinsed with PBT (PBS, 0.1% Tween 20) before proteinase K treatment was begun. Hybridization with *ZAM env* or *pol* digoxigenin-labeled RNA probes was performed at 55°C overnight and was followed by washes in 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

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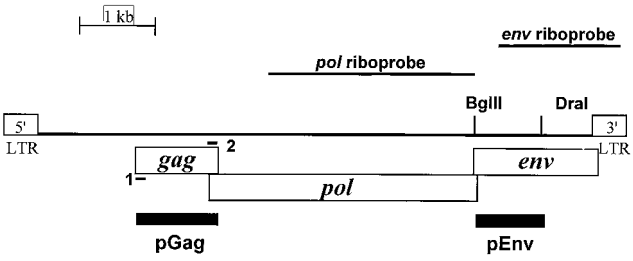

FIG. 1. Genetic organization of the *ZAM* retroelement. The retrovirus-like *gag*, *pol*, and *env* ORFs are flanked by 5′ and 3′ LTRs. The *pol* and *env* riboprobes used for in situ hybridization experiments are shown, as are *ZAM* polypeptides used for preparing polyclonal antibodies directed against Env and Gag proteins (pEnv and pGag, respectively). 1 and 2, oligonucleotides used for the PCR amplification of the *gag* ORF. The *Bgl*II and *Dra*I restriction sites in the *env* ORF are those used for subcloning the envelope-encoding region into the pRSETC expression vector.

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 Genius 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 AACAACTCGCTCCGTGTTA-3′; positions 1819 to 1839) and oligonucleotide 2 (5′-GGAATTCCTTCTATGTTGTGTAGCCC-3′; positions 2805 to 2823) (Fig. 1). Oligonucleotides 1 and 2 display at their 5′ ends *Bgl*II and *Eco*RI restriction sites, respectively. The *gag* PCR product was inserted into the pGEX4T2 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 *Bgl*II-*Dra*I DNA fragment (Fig. 1) encoding the N-terminal part of *ZAM* Env protein was subcloned from the BH clone (11) into the pRSETC 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 by chromatography on a nickel affinity resin (Invitrogen).

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

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 and copiously rinsed in 1× PBS. Immunodetections were performed with the ABC-Vectastain kit (Vector Biosys) according to the manufacturer protocol. Primary antibodies (pAbGag or pAbEnv) were added at 1/1,000 dilution. Preadsorbed secondary antibodies (goat anti-rabbit horseradish peroxidase (HRP) or goat anti-rat HRP) were added at a dilution of 1/400. After coloration ovaries were analyzed by optical microscopy (Zeiss Axiophot microscope).

For fluorescence stainings, we have used fluorescein isothiocyanate-conjugated antirat 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. Following a prolonged rinse in the same buffer, the ovarian follicles were postfixed 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 eventually 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 resins and allowed to polymerize under a UV lamp at 4°C for 3 days. Sections were obtained with an LKB ultramicrotome and mounted over 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 (pAbGag) or rat (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 antirat (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 2; 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 w^{IR6} 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 long 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 w^{IR6} 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 w^{IR6} testes or in w^{IR6} 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 germarium. 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 germarium 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 germarium 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

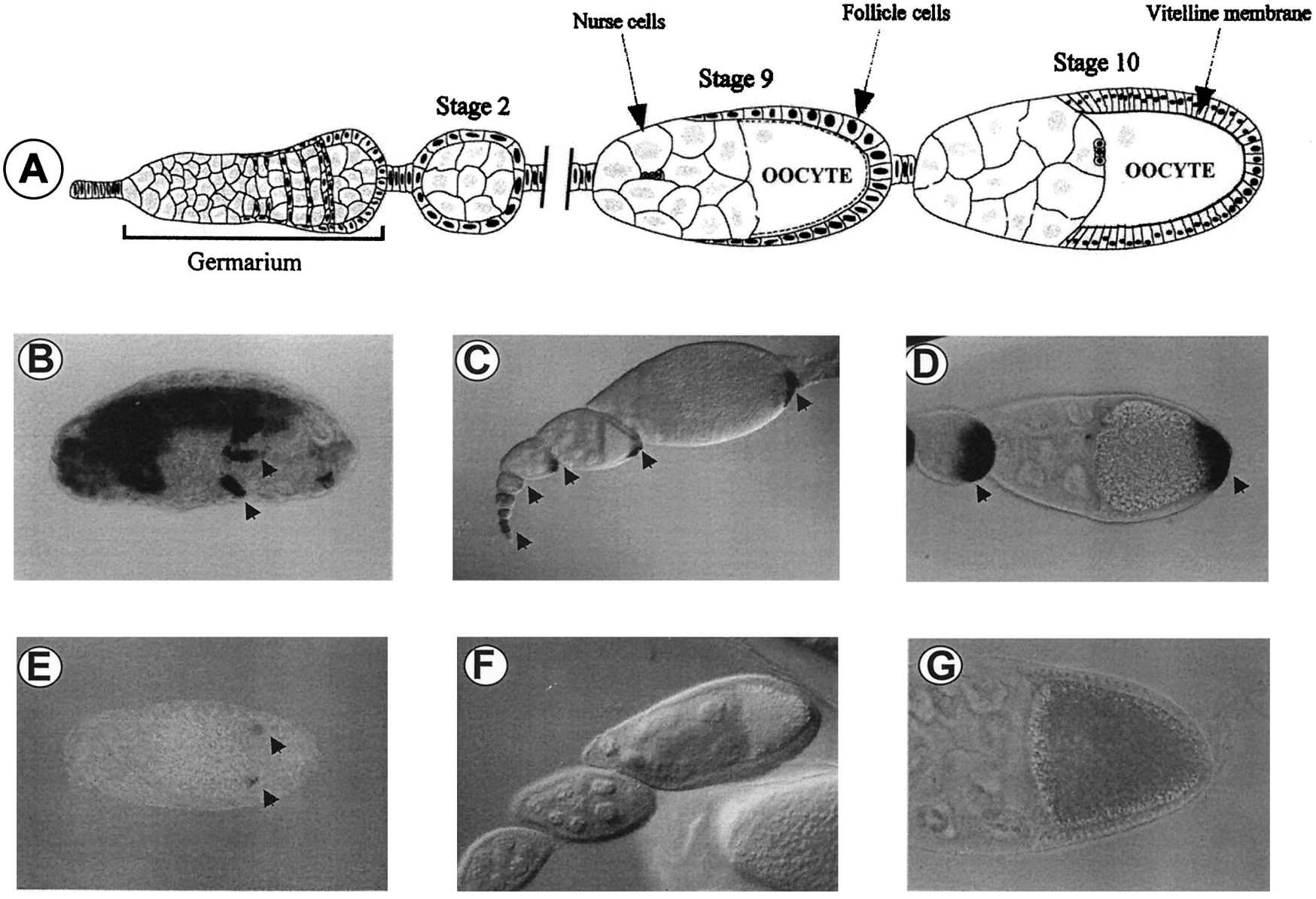

FIG. 2. In situ hybridization to whole-mount embryos and ovaries to visualize the distribution of *ZAM* transcripts. (A) Schematic representation of an adult ovariole. The ovariole is composed here of the germarium (early stages of oogenesis) and later of two follicles in stages 9 and 10. The germ line 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 w^{IR6} strain. After a long time of revelation, a leaky signal is observed in the gonads (arrowheads). (F) Ovariole of the w^{IR6} 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.

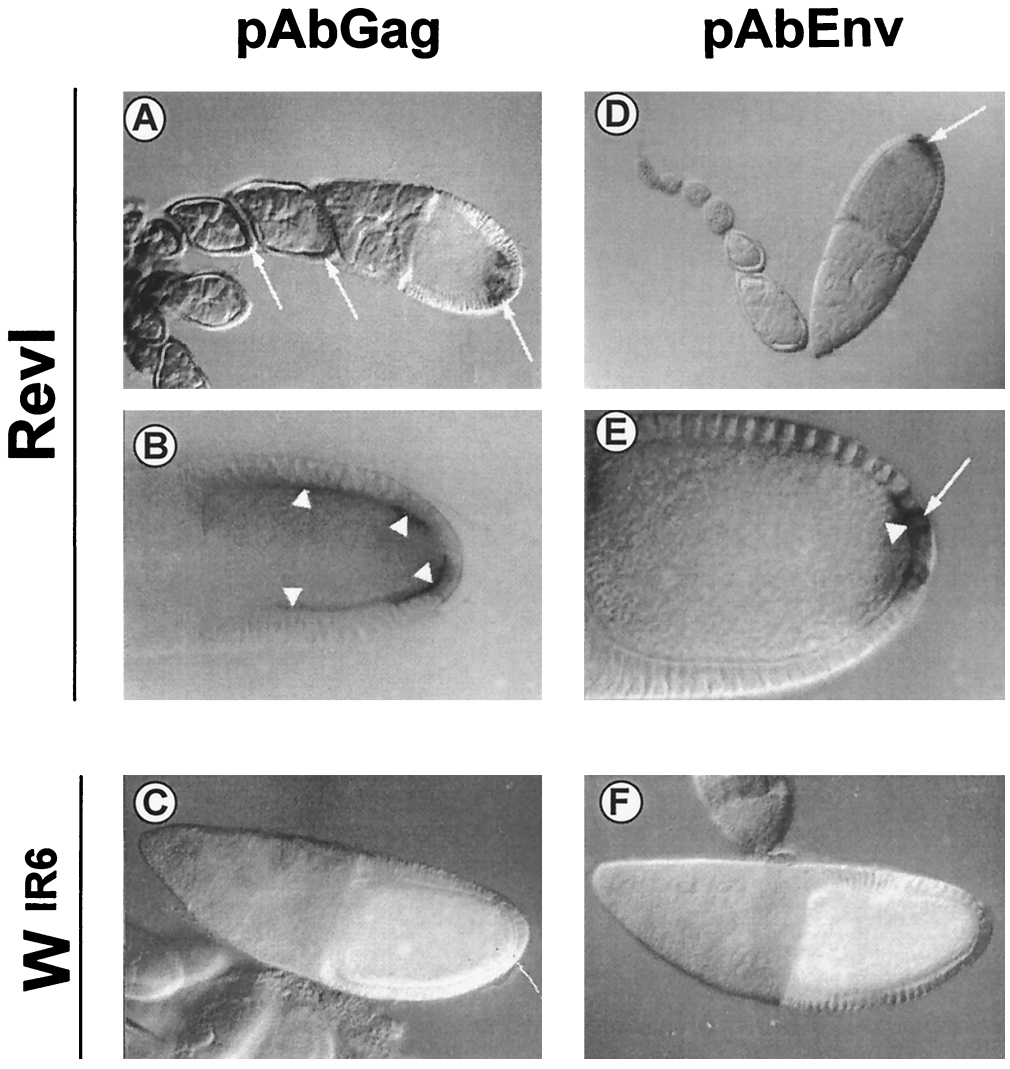

FIG. 3. Expression of Gag and Env proteins during *Drosophila* oogenesis. Shown is the immunolocalization of Gag and Env proteins using polyclonal pAbGag and pAbEnv antibodies, respectively, in ovaries of *RevI* and w^{IR6} females. (A to C) Gag of *ZAM* revealed with the pAbGag antibody in a *RevI* 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.

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 polypeptides 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

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^{IR6} 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 extended from nucleotide 6385 to 7105 of *ZAM* ORF3, was synthesized (pEnv; Fig. 1). After purification by chromatography on a nickel affinity resin, it was injected into rats (see Materials and Methods). A polyclonal antibody denoted pAbEnv, which recognizes the recombinant histidine-ORF3 peptide in Western blot analyses whereas the preimmune serum does not, was obtained (data not shown).

Using pAbEnv, we then determined the temporal and cell type-specific expression of *ZAM* Env protein in flies. No immunoreactivity was detected with pAbEnv in embryos or larvae (data not shown). Env proteins revealed with pAbEnv were only detected in the ovaries of the *RevI* strain (Fig. 3D and E). This translation of *ZAM* ORF3 was restricted to the very small patch of follicle cells surrounding the oocyte where *ZAM* transcripts were identified. However, the signal was detected in a more defined subset of these somatic cells since it was present at the posterior parts of stages 9 and 10 and not at earlier stages of follicle development. At a higher magnification a strong immunostaining highlighted the area along the follicle cells and the oocyte in addition to Env presence inside the follicle cells (E).

In order to verify the specificity of this immunostaining, controls were performed using the pAbEnv antibody and the preimmune serum on w^{IR6} and *RevI* ovaries, respectively. No Env protein was detected in ovaries of the w^{IR6} strain (Fig. 3F), and no immunostaining was observed with the rat preimmune serum on *RevI* ovaries (data not shown).

Gag and Env proteins encoded by *ZAM* are coexpressed in the same somatic lineage. To verify that Gag and Env proteins are indeed coexpressed in the follicle cells of *RevI* egg chambers, we used confocal microscopy on *RevI* ovaries stained with anti-Gag antibody and anti-Env antibody (Fig. 4).

As expected, double staining revealed that Gag and Env proteins are coexpressed at stages 9 and 10 A of oogenesis in the follicle cells located at the posterior part of the ovarian follicle (Fig. 4, upper panels). The detected fluorescence is specific to these follicle cells and almost absent within the other follicle cells surrounding the oocyte. At this stage of oogenesis, Gag and Env proteins are visualized within the cytoplasm of the cells.

At a later stage, around stage 10 B, of oogenesis (Fig. 4, lower panels). Gag and Env are detected as a thick line visualized at the boundary of the follicle cells and the oocyte. No Gag or Env signal was clearly detected further within the ooplasm of the oocyte or in the nucleus, which is located at the opposite side of this germ cell. At this stage of development, an Env signal persists within the follicle cells while the Gag product is no longer visualized. This picture of the Gag signal detected within the follicle cells and then concentrated at the follicle cell-oocyte border at a later stage of development is consistent with movement of Gag-containing particles between the two.

Viral particles are detected in the follicle cells of the *RevI* line. Since all components necessary to assemble particles have been found in a small patch of cells clearly identified, we then searched for potential *ZAM* particles by an electron microscopy approach. A number of ovarian follicles from *Drosophila* strain *RevI* were examined by electron microscopy and compared with those from w^{IR6} flies. Ellipsoidal or ring-shaped particles about 45 nm in diameter with an electron-translucent center were regularly seen in the posterior follicle cells of ovarian follicles at stages 8 to 10. The region of the follicle cell cytoplasm most highly enriched in viral particles is the one close to the apical plasma membrane (Fig. 5A and B). Some particles have also been occasionally observed inside the nucleus.

No viral particle was detected in any of the ovarian tissues or intercellular spaces examined in the w^{IR6} line (Fig. 5C). This result is in good agreement with data presented above concerning the w^{IR6} line in which no *ZAM* mobilization or any *ZAM* RNA or Gag or Env polypeptides have been observed and strongly supports the idea that the presence of the particles detected in *RevI* is correlated with *ZAM* mobilization.

At stages 8 to 10 of oogenesis, *Drosophila* oocytes accumulate large amounts of yolk. Yolk precursor proteins are synthesized in the fat body and are transported via the hemolymph to the oocyte membrane, where they are subsequently taken up by endocytic vesicles. However, a significant amount of yolk protein is also synthesized in the follicle cells themselves (17).

Interestingly, most of 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^{IR6} 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).

Viral particules were also detected within the oocyte at stages 8 to 10 of oogenesis. At these stages, the *Drosophila* oocyte is heavily involved in taking up vitellogenin from the hemolymph by receptor-mediated endocytosis (18). The endocytic apparatus at these developmental stages comprises a plethora of vesicles including coated vesicles, transitional yolk bodies, and mature yolk granules (6). Viral particles can be easily recognized by size and shape among these vesicles in the cortical ooplasm of *RevI*. Viral particles were located over the yolk granules, where they appear uniformly dispersed along the superficial layer (Fig. 7C) or enclosed within a vesicular membrane (Fig. 6C and D).

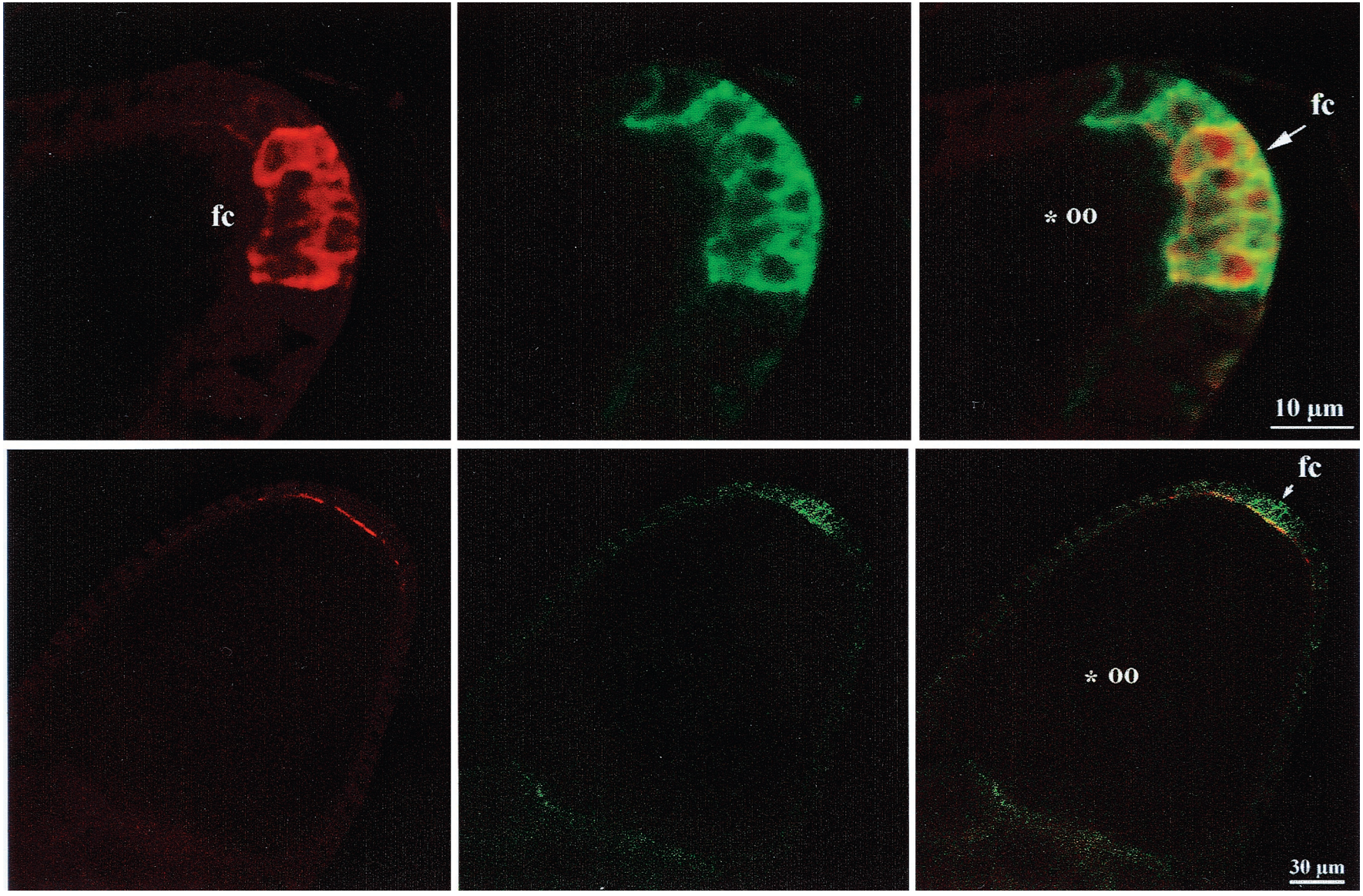

FIG. 4. *ZAM gag* and *env* genes are coexpressed in the follicle cells of *RevI* follicles. Double staining of *RevI* follicles with Gag antibody (red) and Env antibody (green) in early stage 10 of oogenesis (upper panels) and in later stage 10 (lower panels). Bars: upper panels, 10 μm; lower panels, 30 μm. oo, oocyte; fc, follicle cells.

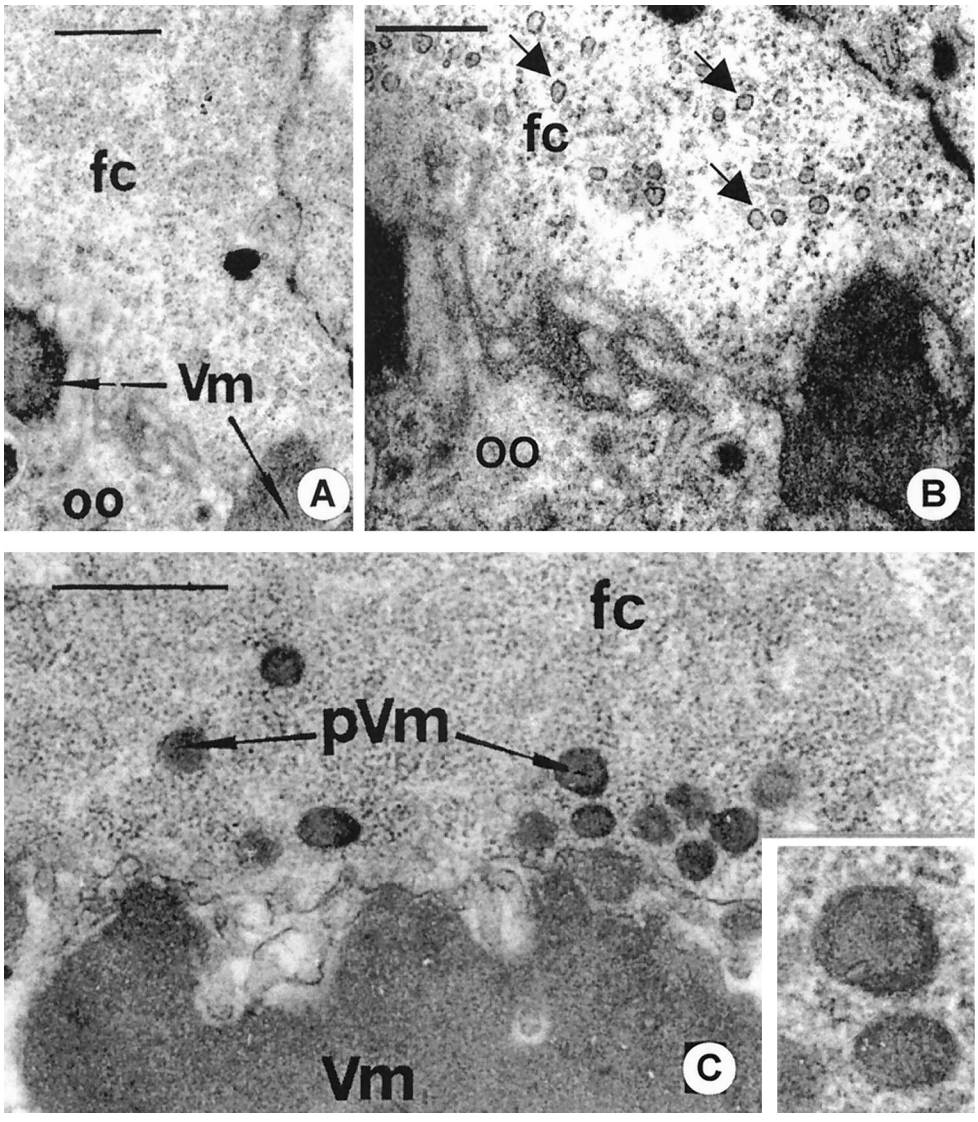

FIG. 5. Ultrastructural identification of *ZAM* viral particles in ovarian follicles of the *RevI* strain from *D. melanogaster*. (A) A posterior follicle cell (fc) facing the oocyte (oo) from a stage 9 ovarian follicle is shown. Vm, vitelline membrane. Bar, 0.5 μm. (B) Enlargement of panel A to show the apical end of a follicle cell, where numerous roundish viral particles (arrows) (average diameter, 45 nm) can be clearly seen. Bar, 0.3 μm. (C) The apical end of a posterior follicle cell from a stage 10 ovarian follicle of the w^{IR6} strain showing several vitelline membrane precursors (pVm) and the vitelline membrane itself, but not viral particles. Bar, 0.9 μm. (Inset) Enlargement of the vitelline membrane precursors.

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

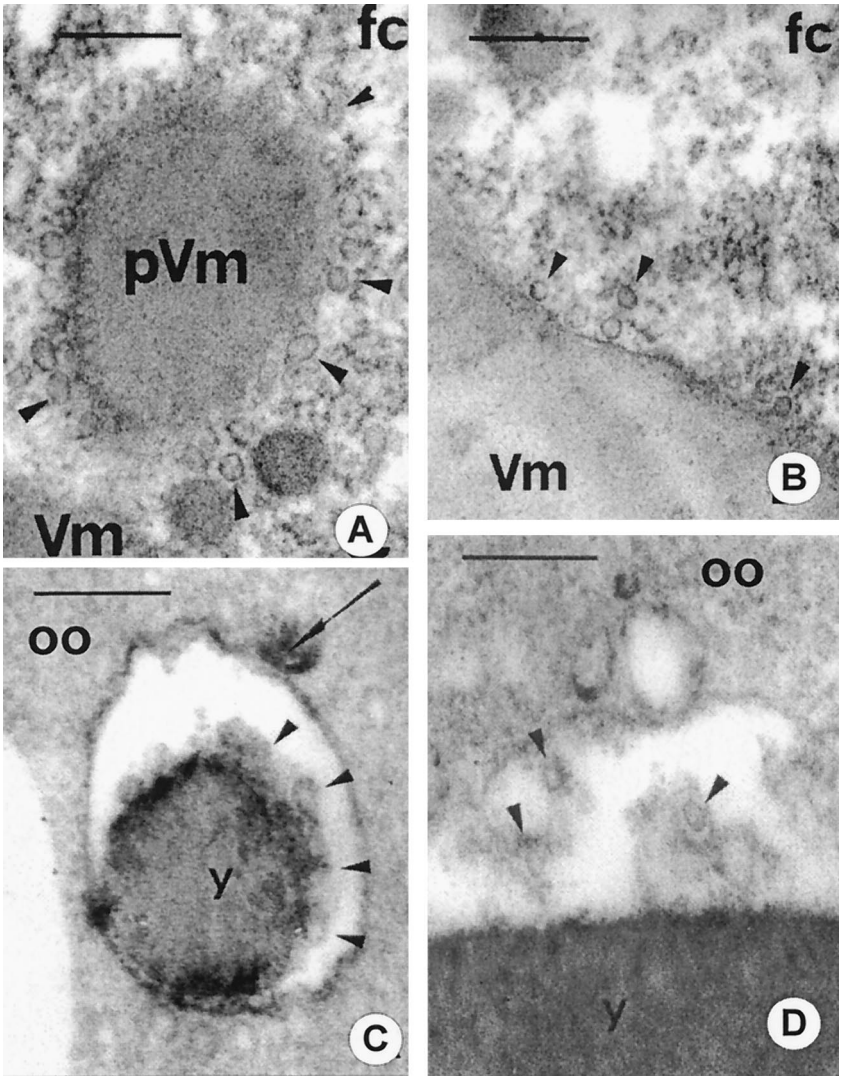

FIG. 6. Cytochemical detection of viral particles on vitelline membrane precursors and in yolk granules of the *RevI* strain. (A) A vitelline membrane precursor (pVm) along the apical end of a posterior follicle cell (fc) from a stage 9 ovarian follicle. Note the presence of numerous viral particles (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 ooplasm 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.

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^{IR6} 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

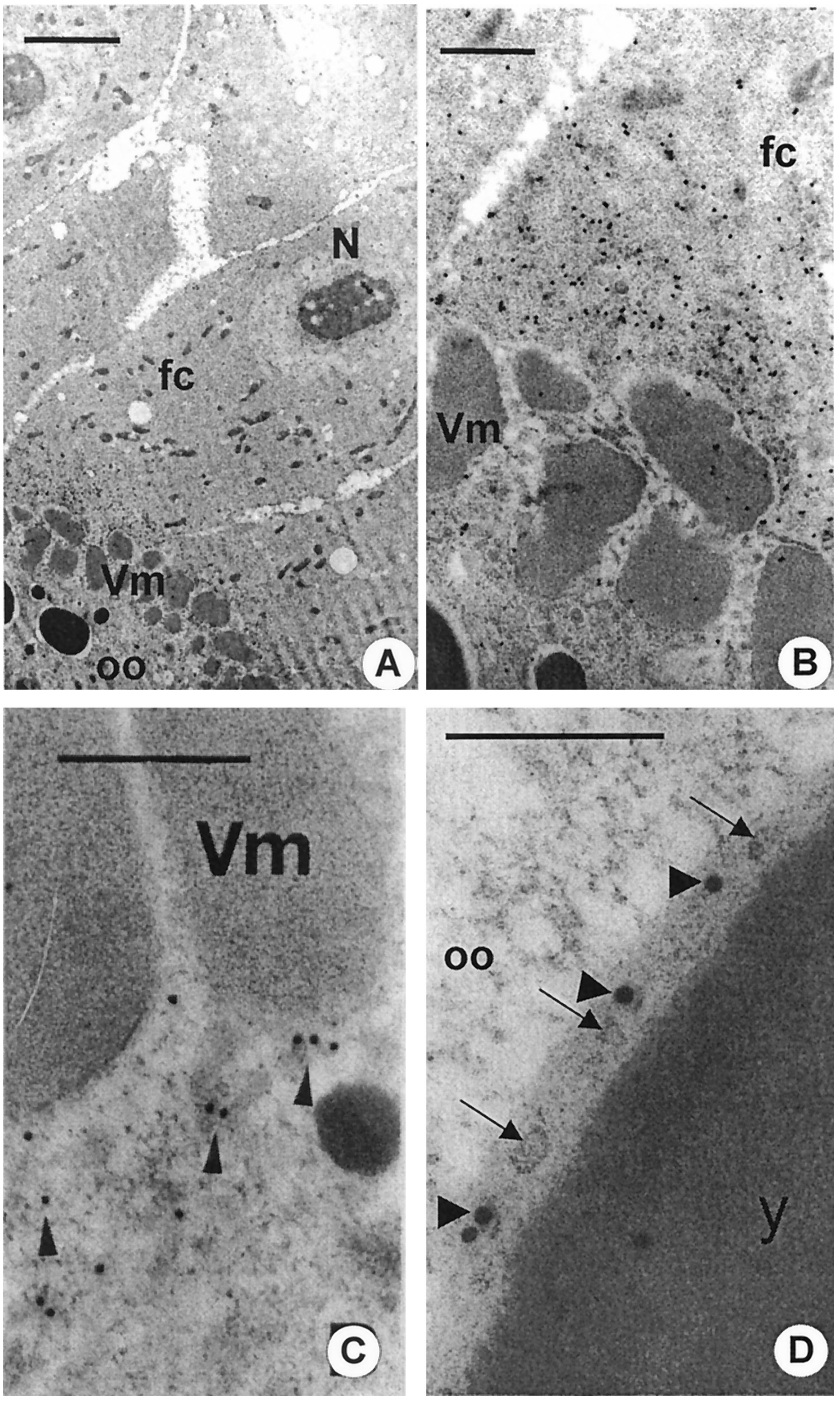

FIG. 7. Immunocytochemical detection of Gag viral antigens. (A) The follicle cell-oocyte border from a stage 9 *RevI* ovarian follicle tested with anti-Gag antibody. fc, posterior follicle cell; N, follicle cell nucleus; oo, oocyte; Vm, vitelline membrane. Bar, 4 μm. (B) Enlargement of panel A to show 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.

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.

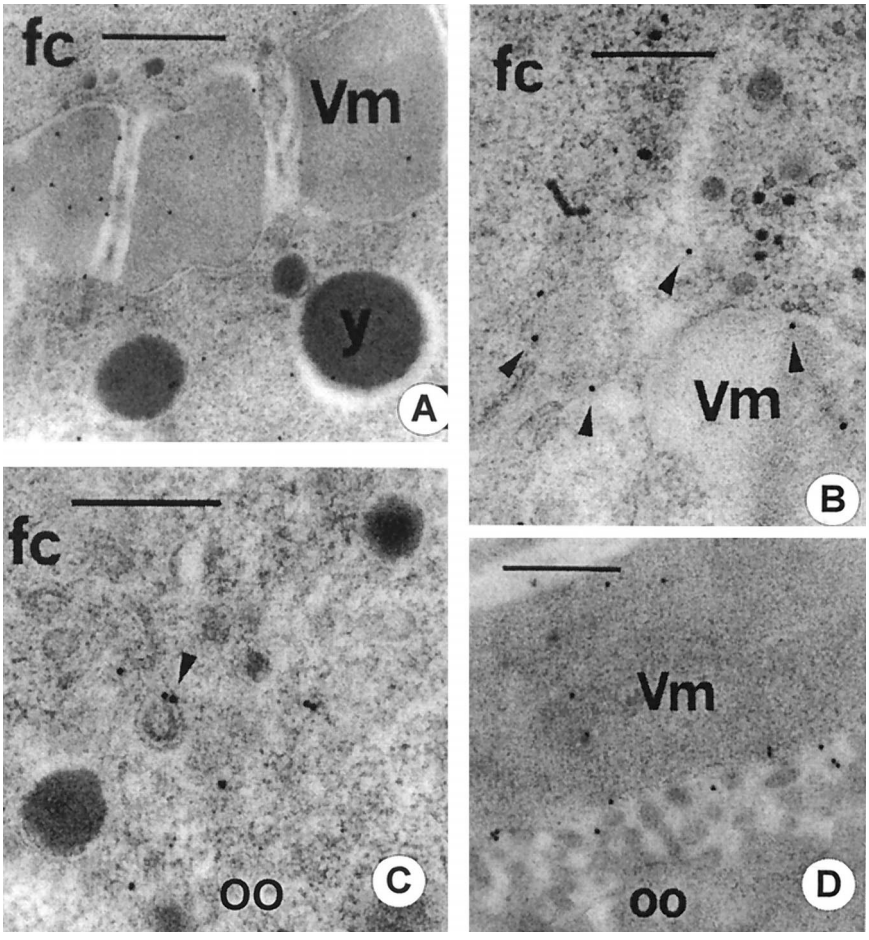

FIG. 8. Immunocytochemical detection of Env viral antigens. (A) The follicle cell (fc)-oocyte border from a stage 10 *RevI* ovarian follicle exposed to anti-Env antibody. Gold grains are dispersed over the vitelline membrane (Vm). y, yolk granule. Bar, 0.5 μm. (B) The apical end of a posterior follicle 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.

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^{IR6}, 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 precursors along the apical cytoplasm 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 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-oocyte 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 posterior 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 along the plasma membranes of the anterior follicle cells. When these cells were tested with anti-Env antibodies, gold labeling appeared almost exclusively associated with the plasma membrane but no viral particle budding or extracellular release 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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