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# Expression of the *Drosophila* retrovirus *gypsy* as ultrastructurally detectable particles in the ovaries of flies carrying a permissive *flamenco* allele

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**The endogenous retrovirus *gypsy* is controlled by the *Drosophila* gene *flamenco* (*flam*). New insertions of *gypsy* occur in any individual *Drosophila* if its mother is homozygous for the *flam*[1] permissive allele and contains functional *gypsy* proviruses. The ovaries of *flam*[1] females also contain high amounts of *gypsy* RNAs. Unexpectedly however, *gypsy* derepression does not occur in the *flam*[1] female germ-line proper but in the somatic follicular epithelium of the ovary. Since extracts from these females are able to efficiently infect the germ-line of a strain devoid of active *gypsy* proviruses, we assume that a similar kind of germ-line infection, which would occur inside the *flam*[1] females themselves, could be required for *gypsy* insertions to occur in their progeny. This hypothesis was confirmed by electron microscopy observations showing that non-enveloped intracytoplasmic particles containing *gypsy* RNAs accumulate in the apical region of the *flam*[1] follicle cells, close to specific membrane domains to which the *gypsy* envelope proteins are targeted, whereas both are absent in the *flam* controls. Low amounts of similar virus-like particles were also observed in *flam*[1] oocytes, but it is not yet known whether they entered passively or as a result of membrane fusion. This is the first report of the beginning of a retrovirus cycle in invertebrates and these observations should be taken into account when explaining the maternal effect of the *flamenco* gene on the multiplication of *gypsy* proviruses.**

## Introduction

A unique feature of retroviruses is their ability to exist both/either as exogenous viruses which, like classical viruses are propagated by horizontal infection, and/or as endogenous viruses which are transmitted vertically in the germ-line where they may behave as reasonably stable Mendelian genes (Coffin, 1990; Lower *et al*., 1996). Endogenous proviruses may account for as much as 1% of the entire genome of *Drosophila* (Bucheton, 1995), mice (Varmus & Brown, 1989) and humans (Lower *et al*., 1996). They are considered to arise from occasional infections of the germ-line by exogenous retroviruses.

However, the manner in which endogenous proviruses gain entry to the germ-line and the mechanisms by which they increase in number (virus replication cycle and/or intracellular transposition) are still unknown because very few model systems have been amenable to full genetic and molecular analysis. For instance, the acquisition by mice of new germ-line C-type proviruses is generally quite a rare event (Coffin, 1990) and, although the frequency of this event could be shown to depend greatly upon the genotype of the host strain, the mouse gene(s) involved have not yet been isolated (Bautch, 1986; Spence *et al*., 1989). The recent discovery that *gypsy* endogenous retrovirus apparently interacts in the same way with its *Drosophila* host might serve as a useful model in this respect (Bucheton, 1995).

Like vertebrate endogenous retroviruses, *gypsy* is transmitted vertically as a small number (usually fewer than five) of functional proviruses inserted at variable sites in the genome of a subset of *Drosophila melanogaster* strains. 'Empty strains', devoid of these proviruses, can be maintained without special care to prevent contagion, which explains why *gypsy* has long been classed as a retrotransposon (Arkhipova *et al*., 1995). However, experimental infection was recently observed by

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growing individuals of an empty strain first in the presence of crude extracts (Kim *et al*., 1994) and later in the presence of virus-like particle (VLP) fractions (Song *et al*., 1994). *Drosophila hydei* cell cultures were also shown to acquire *gypsy* proviruses, probably by infection after incubation with the culture medium of *D. melanogaster* cells (Syomin & Ilyin, 1994). High proportions of individuals rich in *gypsy* proviruses were observed in the progeny of treated flies, indicating a strong tropism of the retrovirus for germ-line cells (Kim *et al*., 1994). These infections occurred under special experimental conditions, but the following genetic data suggest that germ-line infection might also occur under normal conditions and be responsible for the increase of the genomic copy number.

Genetic analysis of an unstable strain of *D. melanogaster* identified the *flamenco* gene on the basis of its ability to repress *gypsy* mobilization (Prud'homme *et al*., 1995). In fact, *gypsy* transposition in a given individual does not depend on the genotype of the fly. It is sufficient that the fly's mother is homozygous for the permissive $flam^1$ allele and contains functional *gypsy* proviruses. At the molecular level, a single restrictive $flam^+$ allele represses accumulation of *gypsy* transcripts that are otherwise observed in the ovaries of the homozygous $flam^1$ mother (Pélisson *et al*., 1994). Unexpectedly however, this repression is observed not in the maternal germ-line proper (nurse cells and oocyte) but in the follicular epithelium of somatic origin that surrounds the germinal cells. Hence the hypothesis that infection by *gypsy* of the germ-line of $flam^1$ females is necessary for new *gypsy* insertions to occur in their progeny. This hypothesis is supported by the isolation of infectious enveloped *gypsy* virions from whole extracts of $flam^1$ females (Song *et al*., 1994) and also by the subcellular polarized localization of *gypsy* RNA and envelope protein (Env) in the follicular epithelium: both signals are restricted to the apical part of follicle cells as if *gypsy* virions were targeted at the apical membranes (Pélisson *et al*., 1994).

In the present study we tested this hypothesis using electron microscopy (EM) techniques. The *gypsy* VLPs were found to accumulate inside $flam^1$ follicle cells close to envelope-containing membranes. Although similar particles were also found in the oocytes we could not demonstrate that they were derived from infection of the germ-line by some of the follicular *gypsy* particles.

## Methods

■ ***Drosophila* stocks.** Flies were maintained on standard Gif medium (Gans *et al*., 1975) at 25 °C. Homozygous $flam^1$ and $flam^+$ females were selected respectively from the *419*(*P*)/*FM7a* and *413*(*NP*)/*FM7a* isogenic stocks previously described (Pélisson *et al*., 1994).

■ **EM.** Ovaries of 2–4-day-old well-fed flies were fixed for 1 h by immersion in 1·6% glutaraldehyde in 0·1 M sodium phosphate buffer (pH 7·2), rinsed for 1 h in 0·2 M of the same buffer, post-fixed with 1% osmium tetroxide for 1 h, dehydrated through a graded series of alcohol concentrations and embedded in Epon Araldite (E. Fullam Co.). Semi-thin sections were stained with 1% toluidine blue in water. Ultra-thin sections were contrasted with uranyl acetate and lead citrate, following standard procedures, and observed under Jeol electron microscopes (100B or 1200).

■ **Enzymatic treatments.** Ovaries were fixed with 4% paraformaldehyde in 0·1 M sodium phosphate buffer and embedded in Unicryl resin (Bio Cell). Ultra-thin sections were treated with either Proteinase K (Sigma) or Pronase (Protease XXV, Sigma), at concentrations of 0·25, 0·50 and 1 mg/ml in distilled water for 1 h at 37 °C, and rinsed with distilled water. Some of these sections were then treated with RNase A (Sigma) at concentrations of 0·25, 0·50 and 1 mg/ml in 10 mM Tris–HCl for 60 min at 37 °C, and rinsed in water. In each case, sections were slightly contrasted with an aqueous uranyl acetate solution, according to standard procedures, before EM observation.

■ **Random priming of the *gypsy* probe.** The probe used was a 6·8 kb *Xho*I–*Xho*I internal fragment of *gypsy* which contains a single copy of every sequence of the element (Marlor *et al*., 1986). The gel-purified fragment (200 ng) was labelled in 20 µl, following the protocol of the Random Primed DNA Labelling Kit (Boehringer Mannheim), except that Bio-16-dUTP was used without dTTP at a concentration of 50 nM. The reaction was stopped by adding 10 mM EDTA and DNA was precipitated overnight at −20 °C. After centrifugation for 1 h, the pellet was dried at room temperature and resuspended in hybridization buffer (Escaig-Haye *et al*., 1992). The probe at a final concentration of 10 µg/ml was stored at −20 °C.

■ **Ultrastructural *in situ* hybridization.** Fixation, embedding and sectioning were as described above. Protease pretreatment (0·2 mg/ml proteinase XXV in distilled water) was performed for 15 min at 37 °C. Denaturation of the probe (in boiling water for 4 min), hybridization (at 37 °C for 3 h) and washing (at room temperature, 2 × 5 min in PBS and 1 × 2 min in distilled water) were done as described by Puvion-Dutilleul (1995) with only slight modifications. Different concentrations of the probe (0·5 and 1 µg/ml) were tested and the results were always the same. Hybridization of the DNA probe with *gypsy* RNA was visualized by a two-step procedure: after preincubation in a 10% solution of heat-inactivated goat serum in PBS–BSA (0·1%) for 30 min, sections were incubated with a 1% solution of rabbit anti-biotin (Enzo) in PBS–BSA (0·1%) for 30 min, and then rinsed with PBS (3 × 5 min); the second incubation was performed for 1 h with a 1% solution of goat anti-rabbit antibody (GAR) in PBS–BSA (0·1%) complexed with either 10 nm or 15 nm gold particles (Bio Cell or Amersham, respectively), and then rinsed with PBS (3 × 5 min) and once in distilled water. Sections were slightly contrasted with an aqueous solution of uranyl acetate for 20 min and rinsed in distilled water.

Several series of controls were carried out: (i) omitting the probe from the hybridization buffer; (ii) using a heterologous probe (biotinylated double-stranded mitochondrial DNA encoding 12S rRNA of *D. melanogaster*; Lécher *et al*., 1996); (iii) treating either before hybridization with RNase A (1 mg/ml in 10 mM Tris–HCl, pH 7·3) or after hybridization with RNase H (1 mg/ml in 20 mM Tris–HCl, pH 7·5) for 1 h at 37 °C; and (iv) treating with the secondary antibody without exposure to the primary antibody.

■ **Immunological methods.** Two anti-*gypsy*-Env monoclonal mouse antibodies (MAbs), clones 7B3 and 8E7 (Song *et al*., 1994), were kindly supplied by V. G. Corces (The Johns Hopkins University, Baltimore, Md., USA). Ovaries were fixed with 4% paraformaldehyde, 0·1% glutaraldehyde in 0·1 M sodium phosphate buffer, rinsed in 0·2 M sodium phosphate buffer, dehydrated through a graded series of alcohol concentrations and embedded in either Unicryl or LR White (London Resin Co.) resins. Ultra-thin sections collected on gold grids were preincubated for 15 min in PBS containing 1% BSA, and then incubated

for 5 h with an equal mixture of the two MAbs (each diluted to 20% in 0·1% PBS–BSA) and rinsed with PBS (2 × 10 min). The second incubation was performed for 1 h with 15 nm colloidal gold-conjugate goat anti-mouse IgG (Amersham) diluted to 2% in 0·1% PBS–BSA, and then rinsed several times with distilled water. All procedures were done at room temperature. Sections were contrasted with an aqueous uranyl acetate solution following standard procedures. Controls were made by treating some sections with the secondary antibody (GAM) without the primary antibody, and other sections were incubated with a mouse MAb raised against a peptide epitope derived from the haemagglutinin protein of human influenza virus (Boehringer Mannheim).

## Results

### Background and focus of the EM observations

The literature on oogenesis in *Drosophila* has been surveyed in several excellent reviews (King, 1970; Lasko, 1994; Mahowald & Kambysellis, 1980; Spradling, 1993). It occurs within ovarioles, which contain a series of progressively older egg chambers classified as a succession of 14 developmental stages. In an egg chamber the germ-line proper consists of a cluster of 16 interconnected sister cells, the posterior-most of which, the oocyte, is the future egg whereas the other 15 differentiate into giant polyploid nurse cells. The egg chamber is completely overlaid by an epithelium of somatic follicle cells. During stages 8–10, the oocyte begins to grow steadily as a consequence of the endocytotic uptake of the yolk, which is shed in the haemolymph by the fat body and the follicle cells. Some products of the nurse cells are also actively transported into the oocyte through the ring canals that interconnect the 16 germ-line cells. During stage 9, the follicle cells which overlay the oocyte become columnar, so that by stage 10A most of the follicle cells form a thick epithelium covering the oocyte. The few remaining follicle cells are stretched over the nurse cells (Fig. 1). From stage 10B until stage 13, the bulk of the nurse cell cytoplasm is rapidly transferred from the squeezed nurse cells into the oocyte.

The *gypsy* RNAs accumulate in the columnar follicle cells of *flam*[1] females (Pélisson *et al*., 1994). The maximum accumulation is observed in the follicle cells which cover the anterior part of the oocyte at stage 10B. At this stage, however, most of the vitelline bodies synthesized by the follicle cells during stages 8–10 are fused together giving rise to the vitelline membrane, forming a barrier against the putatively infecting *gypsy* virions. Most of the EM observations were therefore focused on stages 9–10A, which is the short period when evidence of *gypsy* expression is easy to detect and the vitelline membrane is not yet completely sealed.

Virions were first located by direct ultrastructural observation of the columnar follicle cells, oocytes and posterior nurse cells of 12 *flam*[1] females. The observations were then confirmed and refined by *in situ* hybridization to *gypsy* RNAs and immunodetection of *gypsy* Env proteins. Ten isogenic *flam*$^{+}$ females of the genotype previously described by Pélisson *et al*. (1994) were used as controls.

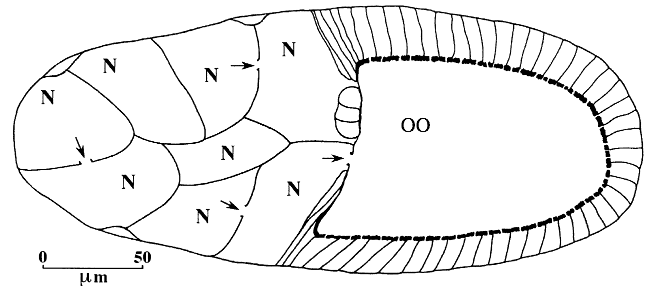


Fig. 1. Schematic drawing of a light micrograph of a longitudinally sectioned stage 10B egg chamber; redrawn from King (1970). Anterior is to the left. Only the cell boundaries are drawn. The thick irregularly dotted line represents the vitelline bodies fusing together to give a continuous vitelline membrane. Only the germ-line cells are lettered; all the other cells are follicle cells. N, nurse cells; OO, oocyte. Note the inward extension of the anterior columnar follicle cells which have started their centripetal migration to cover the anterior part of the oocyte. They intermingle, especially at the level of their apical extensions. Only some of the ring canals, which connect the 16 germ-line cells together, are cross-sectioned (arrows).

### Intracytoplasmic VLPs accumulate close to the membranes in apical regions of the *flam*[1] follicle cells

A specific type of subround particle, shown in Fig. 2, was typically found in every stage 9–10 egg chamber of the 12 *flam*[1] ovaries observed, whereas such particles were not seen in any of the 21 control egg chambers originating from 10 different *flam*$^{+}$ females. These particles were especially abundant inside the anterior columnar follicle cells (Fig. 2*a*), accumulating apically in clusters covering the inner side of the membranes (Fig. 2*b*). At low magnification, these clusters were easily detected as homogeneous areas because they were devoid of organelles (not shown). Moreover, the particles were not in close contact, but were regularly spaced out about half-a-diameter apart.

In spite of the antero-posterior gradient in the follicular epithelium, particles could still be readily found in follicular cells at the posterior poles, especially at later stages (namely stages 11 and 12; data not shown). They were less abundant in younger stages, but some of them could be recognized in the follicle cells of a stage 6 egg chamber (not shown).

Fewer VLPs were detected inside the oocyte (Fig. 2*e*, *f*) and even fewer in the nurse cells (Fig. 2*d*), and there appeared to be no specific localization in these cells. The few small clusters of particles were randomly distributed throughout the hyaloplasm; no specific correlation with the plasma membrane was noticed and association with micropinocytotic vesicles and yolk spheres of the oocyte was rare (data not shown).

These subround particles looked like intracytoplasmic A particles (Bernhard, 1960; Fine & Schochetman, 1978). They had a large diameter of 45 nm and a short one of 40 nm (Fig. 2*c*). They all displayed a ring-shaped structure with the inner side of the ring slightly denser than the outer. The centre of most of them was either homogeneously electron-lucent or had an increasing density towards the periphery.

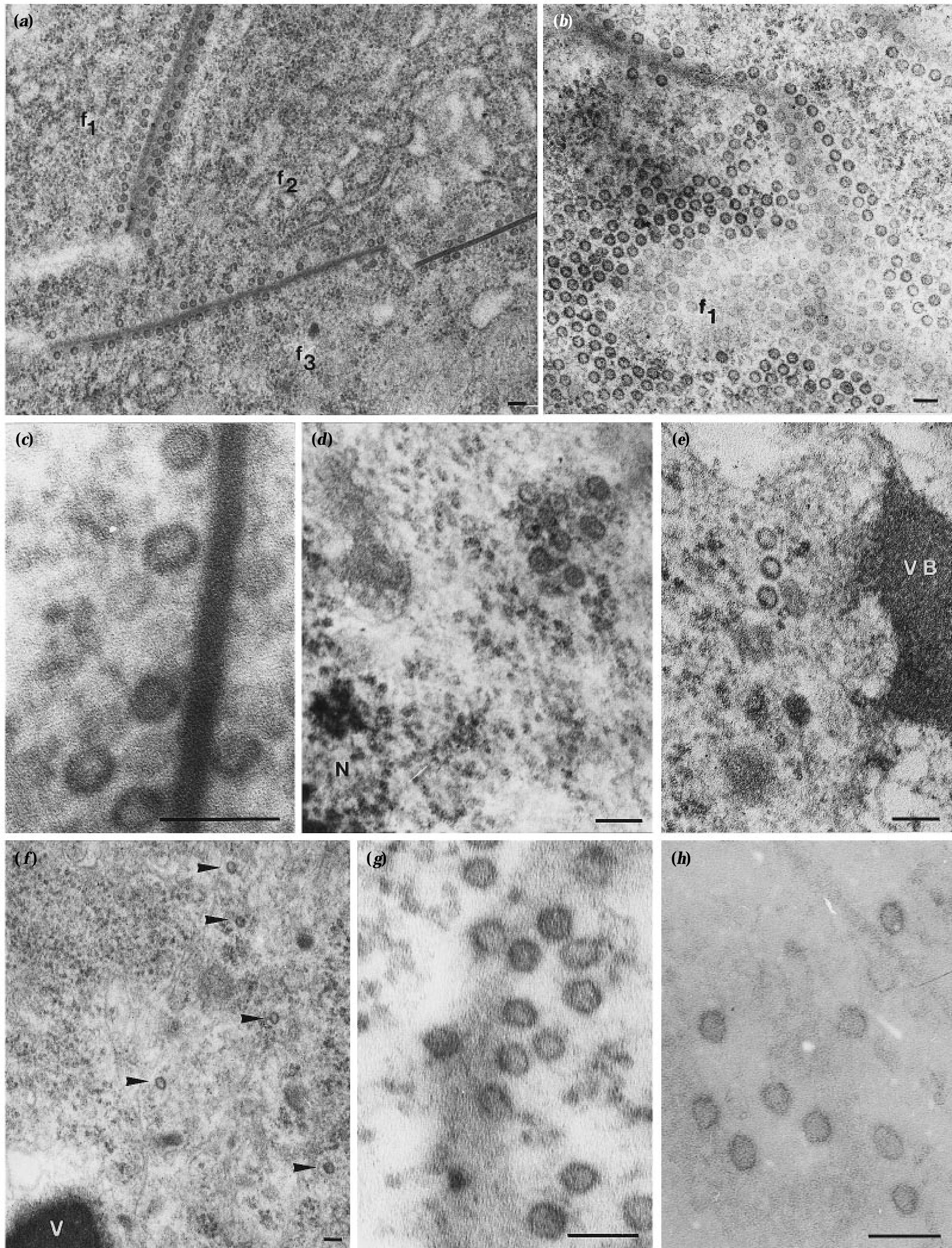


Fig. 2. Localization and ultrastructure of VLPs in *flam*[1] ovaries. (*a*) Apical region of three stage 10B anterior follicle cells ($f_{1-3}$). VLPs are present close to the internal side of the cell membranes. (*b*) As above, except that part of the cell membrane has been cut semi-tangentially to show the cluster of VLPs from above covering the inside of the follicle cell ($f_1$). (*c*) High magnification of VLPs. Their diameter is 40–45 nm. They can be classified as A-type particles. (*d*) One of the very rare clusters of VLPs observed in the cytoplasm of posterior nurse cells not far from the nurse cell nucleus (N). (*e*) Apical part of a stage 10

In order to establish whether any of the electron-dense structures had characteristics of ribonucleoprotein complexes, sections were first treated with increasing concentrations of pronase for 1 h at 37 °C. Concentrations as high as 1 mg/ml did not result in any ultrastructural change (Fig. 2 *g*). Some of these sections were then submitted to an additional treatment with RNase A (1 mg/ml) for 1 h at 37 °C, which, although dramatically affecting the cytoribosomes, only weakly reduced the thickness of the ring-shaped structure of the particles (Fig. 2 *h*). Treatment with higher concentrations of either enzyme was not useful because the cellular structures were destroyed beyond the point at which the landmarks for areas of particle accumulation could still be detected (the membrane is hardly visible in Fig. 2 *h*).

Another significant result was the absence of any extracellular virions. Because of the presence of vitelline bodies between interdigitating microvillar projections it was often difficult to recognize the extracellular space between the oocyte and the follicle cells (see Fig. 4 *b*). Nevertheless, no budding could ever be seen from any of the three cell types in spite of very careful examination and in contrast with the large numbers of intracytoplasmic VLPs in follicle cells.

### Detection of *gypsy* RNA in and around VLPs

In order to find out whether VLPs contain any *gypsy* single-stranded nucleic acid, Unicryl-embedded ultra-thin sections were incubated with a biotinylated *gypsy* full-length DNA, as described in Methods. As previously shown (Pélisson *et al*., 1994), *flam*[1] follicle cells (Fig. 3 *a*, *b*) showed greater labelling than their *flam*$^+$ isogenic counterparts (not shown). Consistent with previous light microscopy observations, the signal was present in the apical part of these cells. Moreover, at the ultrastructural level, the immunogold label was specifically localized over areas of VLP accumulation (Fig. 3 *a*, *b*). In these areas, in addition to the cytosol, about 10% of the VLPs were labelled.

The possibility that the VLP signals were artifacts that could have resulted from occasional VLPs being embedded inside the section just below cytosolic determinants (*gypsy* RNAs or unspecific binding sites) accessible to the probe at the surface of the resin could be ruled out since most of these gold particles specifically labelled the electron-dense ring-shaped structure, which makes up only a small part of the VLP (Fig. 3 *a*, *b*). Hence our conclusion that there were actually two separate signals, one corresponding to the cytosol and the other to the VLPs.

The ability of the immunogold cytochemistry marker technique to label specifically *gypsy* RNA was already suggested as regards the cytosolic signal by its differential intensity between isogenic genotypes (*flam*$^+$ and *flam*[1]), which only differ at the level of *gypsy* expression. This was confirmed by the following controls that were performed to establish the specificity of the VLP signal. The labelling as a whole, including the VLP signal, decreased significantly when sections were treated either with RNase A before hybridization (Fig. 3 *f*) or with RNase H after hybridization (Fig. 3 *g*). Moreover, when either the probe (Fig. 3 *d*) or the anti-biotin antibody was omitted (Fig. 3 *h*) the control experiments did not reveal labelling over VLP areas. The same was true when a 12S rDNA mitochondrial gene (Lécher *et al*., 1996) was used as a probe instead of *gypsy* (Fig. 3 *e*). From these results we can conclude that the areas where the VLPs accumulate are rich in *gypsy* RNAs and that at least some of these VLPs also contain *gypsy* RNAs (see Discussion).

A small amount of labelling was also detected in *flam*[1] oocytes (Fig. 3 *c*). However, in this case it was difficult to get a correct estimation of the percentage of labelled VLPs, and therefore to correlate any of the immunogold label with the VLPs, because they were too few in number.

### The *gypsy* Env proteins are associated with the membranes close to where gypsy VLPs accumulate

Light microscopy techniques using MAbs have shown that *gypsy* Env proteins are also located in the apical part of *flam*[1] follicle cells (Pélisson *et al*., 1994). To gain an insight into the interactions between these proteins and *gypsy* VLPs, their subcellular localization was studied at the ultrastructural level using a 1:1 mixture of anti-Env MAbs as described by Song *et al*. (1994).

In stage 9–10 *flam*[1] follicle cells, the immunogold particles were mostly found apically, labelling the inner side of the membranes (Fig. 4 *a*, *b*). The anti-Env MAbs did not seem to bind to VLPs. Labelling of some VLPs may have resulted from their close proximity to labelled membranes, especially if they were cut tangentially as in Fig. 2(*b*). Indeed, there was always a very good correlation between the labelling of areas of membrane and the presence of *gypsy* VLPs in their immediate neighbourhood. From a practical point of view, this strict correlation suggested that the label was specifically pointing towards *gypsy* Env proteins which may be inserted into these particular membrane domains. Specificity of labelling was further supported by the following observations: (1) only a low-level background could be detected in follicle cells of the

oocyte close to a vitelline body (VB). Some VLPs are present but fewer than are found in follicle cells. (*f*) Part of the hyaloplasm of a stage 12 oocyte with some VLPs (arrowheads); V, vitellus. (*g*) The strongest pronase treatment (1 mg/ml for 1 h at 37 °C), which partly preserves the morphology of cytoribosomes but does not apparently change the ultrastructure of the VLPs. (*h*) The same pronase treatment followed by a similar RNase A treatment (1 mg/ml for 1 h at 37 °C). The cytoribosomes are dramatically affected; the diameters of VLPs are the same as above, but the thickness of the ring-shaped structure is slightly diminished. Scale bars represent 100 nm.

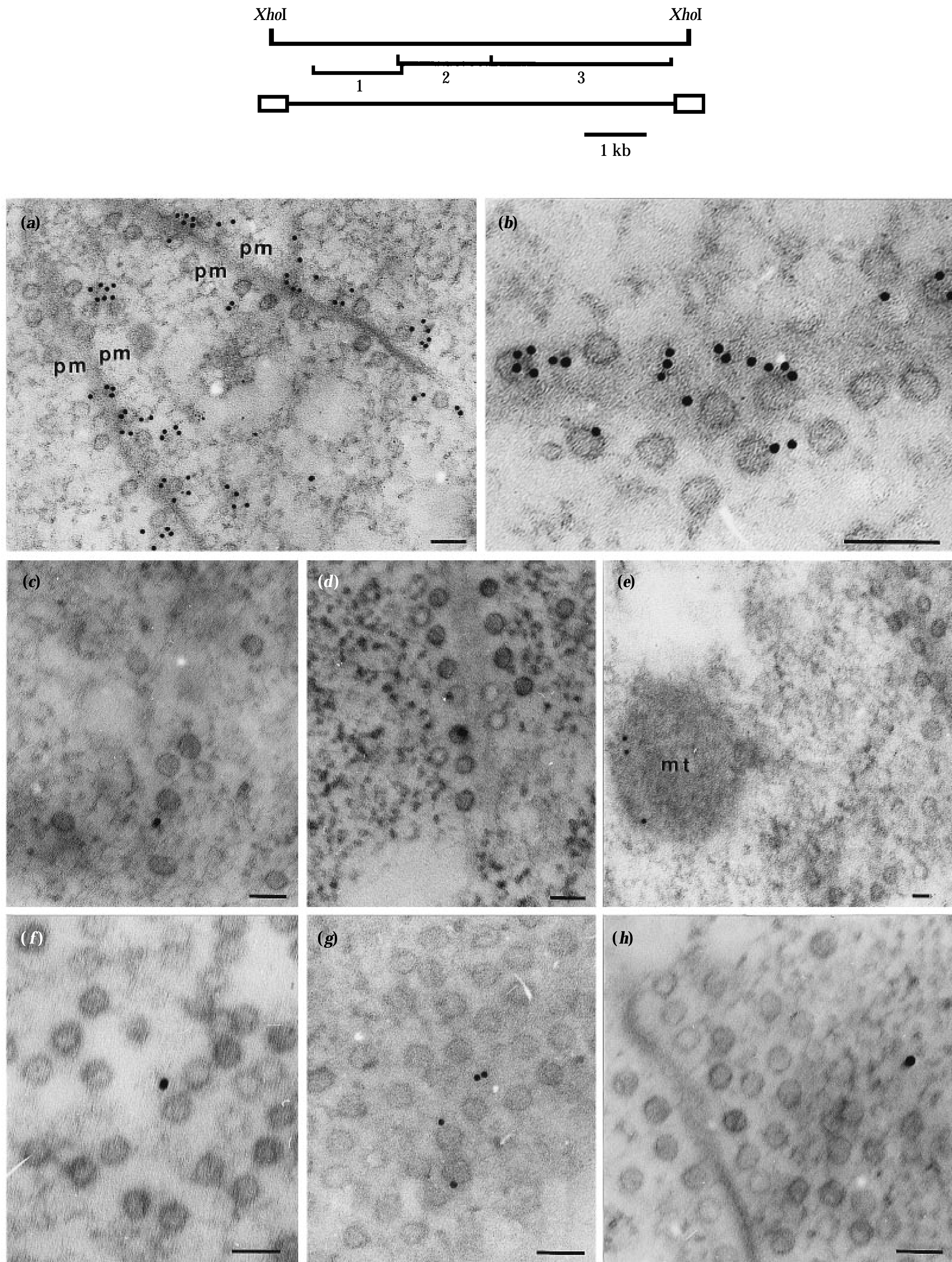


Fig. 3. Ultrastructural *in situ* hybridization of *flam*[1] stage 9–10 egg chambers with a *gypsy* biotinylated probe. Top, the 6·8 kb *Xho*I–*Xho*I fragment used as a probe is shown above a schematic representation of the *gypsy* map with the main body flanked by the two long terminal repeats (open boxes); 1, 2 and 3 indicate the locations of the three open reading frames. (*a*, *b*) *gypsy* RNAs are only detected in the areas of VLP accumulation close to the plasma membranes (pm). Gold particles are often found

*flam*$^+$ isogenic controls (Fig. 4*c*); (2) the same was true when the anti-Env primary antibody was either omitted (Fig. 4*e*) or replaced by an irrelevant MAb against haemagglutinin (Fig. 4*d*).

The immuno-EM revealed a further characteristic of the Env-containing membranes. They appeared to be better preserved by the fixation procedure than either the 'normal' membranes of *flam*$^+$ follicle cells (Fig. 4*c*) or the domains of the *flam*$^1$ follicle cell membranes, which are devoid of Env and close to which no VLPs are seen (Figs 2*a* and 4*a*, *b*). No significant Env label was detected in the *flam*$^1$ germ-line cells. Only a weak signal was sometimes seen in oocytes but it was impossible to correlate it with the few particles present there (Fig. 4*f*). This is in agreement with the fact that they were devoid of an external envelope-containing shell.

Ten anti-Env-labelled egg chambers from *flam*$^1$ females were also carefully examined for enveloped extracellular particles that might have been overlooked in the previous ultrastructural observations. The fact that no such virion could be detected by envelope labelling strengthened the conclusion that they were either absent or very rare.

## Discussion

This study demonstrates that the expression of *gypsy* proviruses in *flam*$^1$ ovaries results in the accumulation of *gypsy* VLPs inside follicle cells, especially at the beginning of stage 9 of oogenesis. This conclusion is supported by the following three observations. (i) About 10% of these particles hybridized to the *gypsy* probe. This low percentage may be explained in two non-exclusive ways: first, only a small proportion of VLPs would contain *gypsy* RNA and second, viral RNA sequences would not always be accessible to the probe, mainly because they are embedded inside the resin. Similar results were obtained by Escaig-Haye *et al*. (1992) who used the same *in situ* hybridization technique on HIV and found that only 20% of the virions were labelled. The *gypsy* particles are much smaller than HIV virions (at most 45 nm as compared to 110 nm for HIV), which could further decrease the probability of the sections passing through viral RNA. (ii) Their tendency to accumulate close to *gypsy*-Env-containing membranes suggests that these particles are actually products of the *gypsy* retrovirus. (iii) These VLPs were not present in *flam*$^+$ ovaries, the genotype of which is restrictive for *gypsy* transposition; these control females are almost completely isogenic with the *flam*$^1$ females since only the small proximal part of the X chromosome that contains the *flam*$^1$ allele had been eliminated by recombination (Prud'homme *et al*., 1995). Moreover most, if not all, of the active *gypsy* copies are located outside of this region (Prud'homme *et al*., 1995). The absence of *gypsy* VLPs from the *flam*$^+$ control ovaries cannot therefore be explained by a lower number of *gypsy* proviruses but rather by the restrictive action of the *flam*$^+$ allele upon these proviruses.

Small spherical particles about 50 nm in diameter have been described in preparations from *Drosophila* cell culture media that have previously been shown to contain *gypsy* nucleic acids (Syomin *et al*., 1993). These were assumed to result from uncoating of the major type of particles (about 70–75 nm in diameter). However, the specific pattern displayed by the parts of the outer shell that were left has never been described for retroviruses, but is reminiscent of the surface projections, or 'capsid chimneys', observed with uncoated reovirus cores (Teninges *et al*., 1979). Another abundant type of particle, about 100 nm in diameter, has also been extracted from whole females of the same *flam*$^1$ genotype as those studied here (Song *et al*., 1994). This size discrepancy is still open to discussion and may be explained either by some sort of swelling occurring during the isolation process or by the fact that the particles detected in this study were devoid of the envelope detected in the latter.

Another significant result from this study was that no enveloped *gypsy* particle was ever found in stage 9–10 egg chambers of these females. The tissue producing the infectious enveloped particles obtained from whole female extracts (Song *et al*., 1994) is therefore still unknown. The *gypsy* Env proteins were only detected in specific membrane domains of the follicle cells and neither budding nor extracellular particles were observed. Whether this retrovirus replication cycle is completely abortive is discussed below.

The most puzzling question remains the molecular basis of the maternal effect of the *flamenco* gene upon *gypsy* transposition. According to Prud'homme *et al*. (1995) and Prud'homme (personal communication), derepression of active copies of *gypsy* in *flam*$^1$ females is necessary to induce transposition in their progeny. The present *in situ* hybridization results show that there is very weak, if any, *gypsy* expression in the *flam*$^1$ female germ-line proper (nurse cells), confirming previous light microscopy observations where this small

outside the VLPs in the cytosol. The gold particles that label VLPs are mostly concentrated on their external ring-like structure. (*c*) In the oocytes a faint signal may be present near VLPs, but both are too rare for any significant association between them to be firmly established. (*d–h*) Micrographs of follicle cells obtained from the five following control experiments: (*d*) the normal experimental protocol was followed, but the probe was omitted from the hybridization solution – only faint labelling was observed; (*e*) a heterologous probe (double-stranded mitochondrial DNA encoding 12S rRNA of *D. melanogaster*) was used – the expected signal was obtained on mitochondria (mt) and VLPs were unlabelled; (*f*) RNase A treatment before hybridization reduced the labelling to background level (as a control for efficiency of the treatment, the cytoribosomes are degraded); (*g*) RNase H treatment after hybridization partially reduced the labelling; (*h*) sections were treated with the secondary antibody without exposure to the primary antibody – only a few gold particles are present. The size of the gold particles was either 10 nm (*a–c*, *e*, *g*) or 15 nm (*d*, *f*, *h*) in diameter. These were used as size standards to confirm the size of the particles (see Fig. 2*c*). Scale bars represent 100 nm.

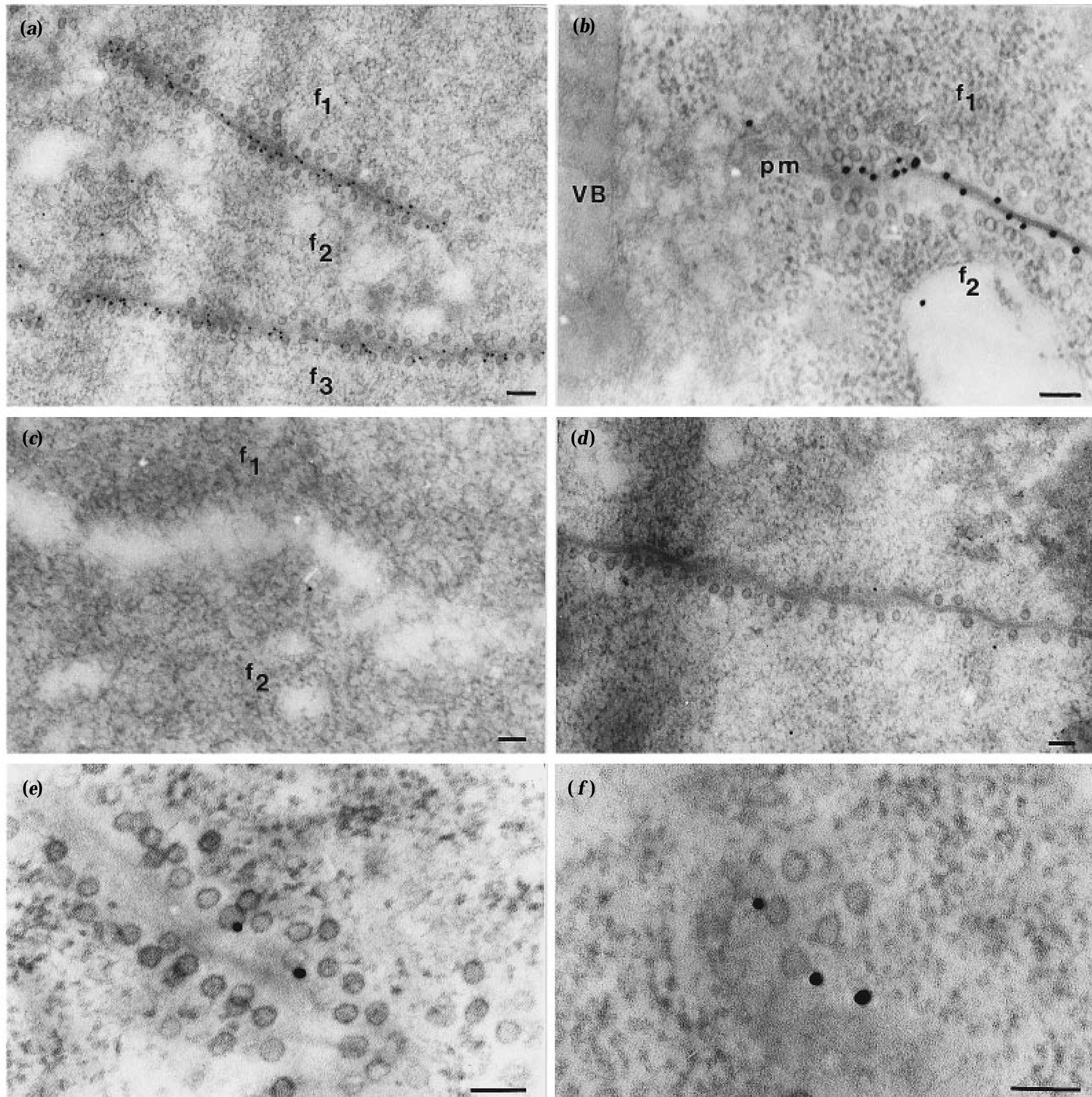


Fig. 4. Immuno-EM localization of the *gypsy* Env proteins in stage 10 egg chambers from either *flam*$^1$ (*a*, *b*, *d*–*f*) or *flam*$^+$ (*c*) females. (*a*) Low magnification of the apical region of three stage 10A anterior follicle cells ($f_{1-3}$). Anti-Env MAbs specifically react with those domains of the membranes close to which VLPs accumulate. (*b*) Higher magnification of the apical region of the boundary between two columnar follicle cells ($f_{1-2}$), showing the anti-Env label at the internal side of both plasma membranes (pm). VB, vitelline body. (*c*) Boundary between two stage 10 *flam*$^+$ anterior follicle cells ($f_{1-2}$). Only a very low level background is detected. (*d*, *e*) Two controls showing the specificity of labelling: follicle cell sections were treated either with the irrelevant anti-haemagglutinin MAb (*d*), or with the secondary antibody (GAM) alone (*e*); in neither case was any significant label detected. (*f*) VLPs in a stage 10 oocyte. Only a very weak signal was detected (see Results). The size of the gold particles was either 10 nm (*a*, *c*, *d*) or 15 nm (*b*, *e*, *f*); these particles were used as size standards to confirm the size of the particles. Scale bars represent 100 nm.

amount of RNA was not found to be significantly higher than that observed in a *flam*$^+$ background (Pélisson *et al.*, 1994). *Flam*$^1$ oocytes were found to contain VLPs; by contrast, none have ever been observed in the *flam*$^+$ control oocytes. Their subsequent integration during embryogenesis might cause the high frequencies of *gypsy* transposition observed in the progeny. However, both the origin of these particles and the way they enter the *flam*$^1$ oocytes are still open to discussion. Even though direct evidence was not found for any obvious infection of these oocytes by the *gypsy* particles assembled in

the neighbouring *flam*[1] follicle cells, it might not be possible to completely rule out the infection hypothesis using EM only; the extracellular step could be transient and too quick to be detected by this technique. Alternatively, non-enveloped particles might enter the oocyte through some unknown cellular process. Such a pathway could consist of endocytotic co-uptake with the yolk; non-enveloped particles could be shed in the haemolymph either by the yolk-producing-tissues (fat body and/or follicle cells) or by some unknown tissue. However, we were unable to detect particles in the micropicnotic yolk vesicles. Whatever the hypothesis, the incoming *gypsy* particle might escape detection by *in situ* hybridization if, immediately after it enters the oocyte, it undergoes reverse transcription so that it can no longer hybridize to the probe (the product of this hypothetical reverse transcription, double-stranded DNA, would not be detected by the method used here).

Even though our observations could not provide evidence that *gypsy* expression in the follicle cells is followed by completion of a full retrovirus replication cycle, the specific apical localization of *gypsy* VLPs is reminiscent of the polarized release of viruses in epithelial cells (Tucker & Compans, 1993). For instance, the VLPs accumulating in the apical region close to the envelope-containing membrane domains could correspond to immature precursors of B- or D-type retroviruses blocked before the final budding step of the retrovirus cycle (Bernhard, 1960; Fine & Schochetman, 1978). The areas of VLP accumulation are also enriched in free *gypsy* RNAs. Whatever the target may be, targeting seems to operate on individual *gypsy* components, as is the case for C-type retroviruses, and not on whole particles that, like B- and D-type retroviruses, would assemble anywhere in the cytoplasm (Rhee & Hunter, 1987, 1990). It should be noted, however, that this is a minor difference since a single substitution within the matrix protein of Mason–Pfizer monkey virus, a D-type retrovirus, can convert its morphogenesis to that of a C-type retrovirus (Rhee & Hunter, 1990). As regards the site(s) of synthesis of *gypsy* individual components, our techniques are probably not sensitive enough to detect *gypsy* RNAs and proteins if distributed at low concentrations in polysomes scattered all over the cytoplasm.

As a typical retroviral *env* product the *gypsy* Env polypeptide seems to be cleaved into a surface (SU) and a transmembrane (TM) protein (Pélisson *et al*., 1994; Song *et al*., 1994). The anti-Env MAbs mostly react with the internal face of the membranes which suggests that an epitope of the short COOH terminus of the TM protein is recognized. This is in agreement with previous results showing that one of the two MAbs, 7B3, reacts with a protein of 28 kDa assumed to be the TM polypeptide (Song *et al*., 1994).

In conclusion, these results provide additional circumstantial evidence that the endogenous retrovirus *gypsy* may take advantage of its infectious potential to multiply in the germen without having to replicate inside germinal tissues, but inside a terminally differentiated tissue instead. Some kind of germinal infection, probably related to the one observed in the more 'artificial' conditions of the experiments reported by Kim *et al*. (1994) and Song *et al*. (1994), would then be responsible for the transposition which occurs in 'natural' conditions.

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