Proviral amplification of the *Gypsy* endogenous retrovirus of *Drosophila melanogaster* involves *env*-independent invasion of the female germline

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***Gypsy* is an infectious endogenous retrovirus of *Drosophila melanogaster*. The *gypsy* proviruses replicate very efficiently in the genome of the progeny of females homozygous for permissive alleles of the *flamenco* gene. This replicative transposition is correlated with derepression of *gypsy* expression, specifically in the somatic cells of the ovaries of the permissive mothers. The determinism of this amplification was studied further by making chimeric mothers containing different permissive/restrictive and somatic/germline lineages. We show here that the derepression of active proviruses in the permissive soma is necessary and sufficient to induce proviral insertions in the progeny, even if the F₁ flies derive from restrictive germ cells devoid of active proviruses. Therefore, *gypsy* endogenous multiplication results from the transfer of some *gypsy*-encoded genetic material from the soma towards the germline of the mother and its subsequent insertion into the chromosomes of the progeny. This transfer, however, is not likely to result from retroviral infection of the germline. Indeed, we also show here that the insertion of a tagged *gypsy* element, mutant for the *env* gene, occurs at high frequency, independently of the production of *gypsy* Env proteins by any transcomplementing helper. The possible role of the *env* gene for horizontal transfer to new hosts is discussed.**

Keywords: extracellular replication/*flamenco*/host-retroelement interactions/soma-germline chimera/transposition assay

Introduction

Retroviruses (RVs) are generally considered, after Temin's early insight (Temin, 1980), as having evolved from retrotransposons that acquired an *env* gene, enabling them

to be transferred horizontally through an extracellular infectious step. As reviewed recently (Boeke and Stoye, 1997), phylogenetic data and pedigree analysis also suggest that RVs may sometimes settle in the germline, thereby becoming the progenitors of new families of vertically transmitted endogenous retroviruses (ERVs). The fact that some ERV families are as large as some retrotransposon families shows how successful the adaptation of such elements to the constraints of their new lifestyle inside the germline can be. An as yet unanswered question, however, is to what extent this efficient amplification of endogenous proviruses involves the primitive mechanism of intracellular retrotransposition. This mechanism might indeed be a default pathway, since both an endogenous (intracisternal A particle, IAP) and an exogenous (Moloney murine leukemia virus, Mo-MuLV) mouse retrovirus were actually shown to be able to transpose, although at very low frequencies, inside somatic tissue culture cells after their *env* gene had been removed (Heidmann and Heidmann, 1991; Tchenio and Heidmann, 1991). However, the full biological significance of such observations would require definite evidence for ERV expression in germ cells. In contrast, the few mouse ERVs whose amplification could be followed, all seem to do so by virtue of the early embryo being reinfected by the viraemic female reproductive tract (Quint *et al.*, 1982; Lock *et al.*, 1988). *Drosophila* is another good model to study how far the endogenization process may proceed. *Drosophila* was indeed recently reported to harbor several families of actively transposing ERV-like elements, including *gypsy* (Pelisson *et al.*, 1994), *Tom* (Tanda *et al.*, 1994) and *ZAM* (Leblanc *et al.*, 1997).

The genomic structure of the *gypsy* retroelement in *Drosophila melanogaster* is remarkably similar to the proviral form of vertebrate retroviruses (Figure 1A). This similarity was even strengthened when it was first shown to be infectious (Kim *et al.*, 1994a). *Gypsy* potentially encodes all necessary *cis*- and *trans*-acting sequences required for infectivity. Among them are the two open reading frames (ORFs) homologous in sequence and organization to the *gag* and *pol* genes of retroviruses (Marlor *et al.*, 1986), and a third one which is expressed via a spliced subgenomic RNA to produce an Env-like protein (Pelisson *et al.*, 1994; Song *et al.*, 1994, 1997). The latter was recently shown to be functional, since it was successfully used to pseudotype a Mo-MuLV and make it infect *Drosophila* culture cells (Teyssset *et al.*, 1998).

Like any endogenous retroelement, *gypsy* moves infrequently in the genome of the host (Nuzhdin and Mackay, 1995; Dominguez and Albornoz, 1996), but a few unstable strains have been described in which it transposes at an unusually high frequency (Kuhn, 1970; Lavery and Lim, 1982; Gerasimova *et al.*, 1984a,b; Mevel-Ninio *et al.*,

Fig. 1. Structure and expression of the *gypsy* constructs. (A) Organization of the *gypsy* element and the Act-env construct. Numbers refer to *gypsy* nucleotides (Marlor *et al.*, 1986). Boxes correspond to ORFs. Two long terminal repeats (LTRs), shown by right-pointing arrowheads, flank a central region which contains the three ORFs corresponding to *gag*, *pol* and *env*. Above this map of the *gypsy* provirus, a close-up of the DNA splicing is represented, which was performed to clone the *env* gene under control of the actin5C promoter: intronic sequences, including the intronic part of the 5' (SD) and 3' (SA) splice sites, were precisely removed, leaving only the last 34 nucleotides of the 5' untranslated exon (nucleotides 535–568) fused to ORF3 and the downstream LTR. SU and TM are the surface and transmembrane subunits of the Env polypeptide. Broken arrows and asterisks indicate the start sites of transcription and the polyadenylation signal, respectively. (B) Structure and expression of the *gyp111fs3* and *gyp6Kfs3* constructs. Two different tags of 23 and 29 nucleotides, respectively, were inserted at the same position of ORF3 (downwards arrowhead, position 5926 in Figure 1A), both causing an immediately downstream stop codon by frameshifting. The spliced polyadenylated subgenomic transcript normally used to express the *env* gene is represented with the resulting truncated ORF. The rightwards arrowhead at position 5423 represents the upstream primer, 5423+, used to detect *gyp111fs3* and *gyp6Kfs3* by PCR when combined with the downstream L1- and L5938flag primers, respectively (left-pointing arrowheads). (C) Structure and expression of the *gyp6Kfs1* construct. A four nucleotides duplication introduced at position 1201 (Figure 1A) caused a frameshift resulting in a stop codon located at nucleotide 1368. The full-length genomic transcript normally used to express the Gag and Gag-Pol polyproteins is represented with the resulting truncated ORF. In contrast, the spliced transcript of this construct is expected to express Env quite normally.

1989; Kim *et al.*, 1990). All these exceptions are likely to result from the independent polymorphic distributions of a small number of functional proviruses on one hand, and of the permissive/restrictive phenotypes of the *Drosophila* genomes on the other (Bucheton, 1995; Pelisson *et al.*, 1997). For instance, a genetically permissive strain may be stable provided it is devoid of functional proviruses. When transformed by the P-element technique with a single functional *gypsy* element, the genome of such a strain becomes unstable as a result of the efficient endogenous multiplication of the initial provirus (Kim *et al.*, 1994b). The same outcome is observed when larvae of a permissive 'empty' strain are raised in the presence of extracts of unstable strains (Kim *et al.*, 1994a; Song *et al.*, 1994). High proportions of infected individuals, rich in *gypsy* proviruses, are observed in the progeny of exposed flies, indicating that *gypsy* is infectious and has a very strong tropism for germline cells. Therefore, the *Drosophila* genome appears to control both the horizontal transfer of *gypsy* and the amplification of the vertically transmitted proviruses. We take advantage of the fact that

Drosophila is amenable to detailed genetic and molecular analysis to manipulate both the virus and the host genome in an attempt to gain insight into this unique paradigm of ERV–host interaction.

Genetic analysis of the permissive/restrictive character identified the X-linked *flamenco* (*flam*) gene as a major regulator of *gypsy* activity (Prud'homme *et al.*, 1995). Several spontaneous permissive and restrictive *flam* alleles have been characterized and their genetic interactions partially described (Pelisson *et al.*, 1997; A.Kim, N.Prud'homme and A.Bucheton, unpublished data). Typically, *flam* shows a strict maternal effect on *gypsy* mobilization: offspring of homozygous permissive females have high rates of *gypsy* transposition, irrespective of their own genotype; in contrast, a single restrictive allele in the mother is usually sufficient to prevent transposition in the progeny even if two permissive *flam* alleles were inherited. Moreover, the frequency of transposition decreases as a function of the maternal age, showing that a major determinant is operating during the mother's adult life (Prud'homme *et al.*, 1995).

At the molecular level, proviral mobilization in the progeny of *flam* permissive females correlates with a dramatic *gypsy* derepression in these females, suggesting that it is regulated maternally at the level of *gypsy* expression. However, this regulation does not take place in the tissue that might be predicted from the maternal effect, the female germline, but in the somatic follicle cells which surround this tissue instead (Pelisson *et al.*, 1994); hence the possibility that *gypsy* mobilization would require the maternal transmission of some *gypsy* encoded material(s) produced in the soma and somehow transferred to the maternal germline. Because of the many similarities with the examples of mouse ERVs amplification mentioned above, a germline infection model was put forward to explain the copy number increase of *gypsy* proviruses (Pelisson *et al.*, 1994; Song *et al.*, 1997).

The objectives of the present study are 2-fold: (i) to understand the basis of the maternal effect of the *flamenco* gene by characterizing the cell lineage where *gypsy* expression is required for its mobilization; and (ii) to ask whether this proviral amplification requires the expression of the *gypsy env* gene. The results clearly show that, unlike bona fide retrotransposons, *gypsy* does not rely on germline expression for its mobilization. In contrast, the expression of a marked provirus in the maternal soma was shown to be necessary and sufficient for its subsequent integration into the germline of the progeny. However, this soma-towards-germline transfer appears to be independent of the expression of the *gypsy env* gene, which suggests that some non-infectious mechanism is involved in this intriguing replication cycle.

Results

Gypsy transposition requires the presence of functional proviruses in permissive mothers

The maternal effect involved in the regulation of *gypsy* mobilization by *flam* is illustrated by the double requirement for a specific age and genotype of the mother: new *gypsy* insertions are mainly observed in the progeny of young permissive *flam* females (Prud'homme *et al.*, 1995). Two hypotheses can be put forward to account for the

Fig. 2. Occurrence of mutations at the *cut* locus in two isogenic female germ lines which only differed by the sexual origin of their active *gypsy* parental proviruses. Two reciprocal experiments, 1F and 1M, were performed simultaneously. Both G_1 parents shared the same *OR* permissive genotype but, unlike *OR*(520), the *OR*(P) strain was devoid of the active proviruses symbolized by an asterisk. As described (Pelisson *et al.*, 1994), the *FM3* restrictive balancer chromosome was used to prevent the mobilization of these proviruses from transposing in the *OR*(520)/*FM3* stock (G_0). The *M5 ct* balancer chromosome used in G_2 carried the *cut* recessive tester allele. The number of phenotypically *ct* females, the total number of observed G_3 females and the corresponding frequency are given for both experiments. Numbers in parentheses indicate independent clusters of mutations.

maternal effect of the *flam* gene. One possibility is that the restrictive genotype would prevent the synthesis of some *gypsy*-encoded product, the maternal transmission of which would be required for new *gypsy* insertions to occur in the embryo. The alternative is that *gypsy* regulation would not occur in the mothers, but in the F_1 individuals, because they would maternally inherit some repressor encoded by the restrictive allele. For the second hypothesis, the presence of *gypsy* proviruses should not be required in the permissive mothers. The following experiment was therefore set up to test whether zygotic proviruses inherited from the father would be allowed to transpose in a *gypsy*-free permissive maternal lineage.

Homozygous females issued from the *gypsy*-rich *OR*(520)/*FM3* stock were crossed to males of the *OR*(P) permissive stock (see Materials and methods), which does not contain functional *gypsy* proviruses, to produce homozygous-permissive females (G_2 in Figure 2, Experiment 1F). Phenotypically *cut* females were observed in the progeny of these females with a minimal frequency of 10^{-3} . The characteristics of these mutants, i.e. overall frequency and small clustering, fitted perfectly with those of the *gypsy*-induced premeiotic mutability previously described (Prud'homme *et al.*, 1995). In contrast, in the reciprocal cross, where the same set of active *gypsy* proviruses was inherited from the father, only one phenotypically *cut* female was detected out of 8267 G_3 females observed (Figure 2, Experiment 1M). This unique female was sterile and we could not test whether this mutation actually resulted from a *gypsy* insertion into the *cut* locus of a G_2 oocyte. Therefore, if *gypsy* ever transposed when the permissive G_1 mothers were devoid of functional proviruses, the transposition frequency appeared to be at least one order of magnitude higher when they did contain functional proviruses.

Transposition cannot occur in the progeny of permissive germinal clones surrounded by a restrictive somatic background

We then addressed the question of whether this *gypsy* maternal effect would still be observed with mosaic mothers where a *flam*¹/*flam*¹ permissive germline would be surrounded by a *flam*^{ovoD1}/*flam*¹ restrictive soma: no expression of the functional proviruses should be allowed in such females except for hypothetical expression in the germline. Such chimeric females were obtained using the classical technique of mitotic recombination induced by irradiating heterozygous *ovo*^{D1} v *flam*^{ovoD1}/*y* v *f mal flam*¹ female larvae. The *ovo*^{D1} dominant sterile mutant prevents the normal development of the female germline. Either mutational inactivation of this antimorphic allele or its elimination from the homozygous cells generated by mitotic crossing-over are the only events to possibly result in fertile germinal clones. Their origin can be deduced from their genotype as determined by progeny testing. The following rationale allowed us to tell which of 88 irradiated fertile females obtained in this experiment had the expected *y* v *f mal flam*¹/*y* v *f mal flam*¹ clone as a result of mitotic recombination between *flam* and the centromere: (i) *flam* is located at the boundary between the euchromatin and the pericentric heterochromatin of the X chromosome (Prud'homme *et al.*, 1995); (ii) <25% of X-ray-induced mitotic recombination is expected to occur distally to *flam* (Wieschaus *et al.*, 1981); and (iii) the *mal* locus used as a marker in this experiment is distal to *flam*. However, the *mal*-*flam* interval contains <2% of the total X euchromatic DNA (Heino *et al.*, 1994; Hartl and Lozovskaya, 1995) and is therefore expected to undergo no more than 0.5% of the total X-ray-induced recombination. As judged by their pure *y f mal* male progenies, 73 of the 88 irradiated fertile females had undergone mitotic crossing-overs located proximally to *mal*. Therefore, 99% of these germline clones should be homozygous for the *flam*¹ permissive allele.

The level of *gypsy* transposition in the progeny of these 73 chimeric females was monitored using the *ovo*^{D1} inactivation assay described in the Materials and methods. For that purpose, they were crossed with *ovo*^{D1} males and the proportion of fertile female progeny was scored. The results of two independent experiments involving different *gypsy* copy numbers are presented in Table I. They show essentially that a *flam*¹/*flam*¹ permissive female germline is not sufficient by itself to induce the high frequency of *ovo*^{D1} inactivation observed in the progeny of the corresponding non-mosaic permissive control females. Instead, the percentages of fertile daughters were very similar for mosaic females and non-mosaic *flam*^{ovoD1}/*flam*¹ control females, i.e. one order of magnitude lower than for the non-mosaic permissive females. It is worth noting that transposition was never completely repressed, even in the progeny of non-mosaic heterozygous females. This means that the *flam*^{ovoD1} allele, when combined with the permissive *flam*¹ allele, allowed a little *gypsy* activity, the level of which was correlated with the number of active proviruses present in the experiment: up to 2.8% of *ovo*^{D1} reversion was indeed observed in the presence of a high *gypsy* copy number (Experiment 2A). Extrapolation of these results suggests that a completely restrictive soma might reduce mobilization to a very low level. We then

Table I. Frequency of *ovo*^{D1} reversion in the progeny of permissive germline clones

Genotypes	Experiment 2A	Experiment 2B
Restrictive SOMA: <i>ovo</i> ^{D1} <i>v flam</i> ^{ovoD1} / <i>y v f mal flam</i> ^I Permissive GERMEN: <i>y v f mal flam</i> ^I / <i>y v f mal flam</i> ^I	9/498 (1.8)	2/167 (1)
Restrictive CONTROL: <i>ovo</i> ⁰ <i>v flam</i> ^{ovoD1} / <i>y v f mal flam</i> ^I	20/1439 (2.8) ^a	2/831 (0.5) ^a
Permissive CONTROL: <i>y v f mal flam</i> ^I / <i>y v f mal flam</i> ^I	271/1923 (14.1)	183/1712 (10.7)

The germ cells present in mosaic females all belonged to the germinal clones (denoted as 'permissive germen') resulting from X-ray-induced mitotic recombination proximally to the *mal-flam* linkage group (see text). The results of the *ovo*^{D1} inactivation assay (see Materials and methods) are given with percentages in parentheses.

^aPercentages doubled in order to compensate for the inability of the *ovo*⁰/*ovo*^{D1} half of the female progeny to produce functional ovaries even after inactivation of the *ovo*^{D1} allele by *gypsy* insertion (see Materials and methods).

The experiment was repeated twice with two isolates of the *flam*^I permissive stock differing by the number of functional *gypsy* proviruses: additional copies were present on the autosomes of the stock used to perform Experiment 2A.

addressed the question of whether a permissive soma would be sufficient to induce it.

Permissiveness of the soma surrounding a restrictive germline is sufficient to induce the insertion of maternal somatic *gypsy* proviruses in the genome of the progeny

The purpose of the experiment shown in Figure 3 was to look for inheritance of the *gyp111fs3* provirus present in the maternal soma (permissive) but not in the germline (restrictive). This tagged defective element, described in Figure 1B and Materials and methods, could be complemented by functional proviruses also present in the maternal soma. The chimeric females were obtained by microinjecting germ cells of the wild-type OR(R) restrictive empty stock into the posterior pole of either *v flam*^{2G}/*ovo*^{D1} *v flam*^{ovoD1} or *v flam*^{FM7c}/*ovo*^{D1} *v flam*^{ovoD1} mutant embryos (Figure 3, Experiments 3P and 3R, respectively). The *flam*^{2G}/*flam*^{ovoD1} genotype is permissive (see Materials and methods), whereas *flam*^{FM7c}/*flam*^{ovoD1} does not allow *gypsy* mobilization (data not shown). Genotypes of the germinal donor and the somatic recipient could be distinguished by their *v*⁺ and *v* phenotypes, respectively. Most of the 166 G₀ females issued from the injected embryos disclosed the expected *ovo*^{D1} sterility phenotype (lack of ovaries): only one of these *v* females produced a *v* progeny, which corresponds to the very low level of *ovo*^{D1} inactivation expected with the completely restrictive G₁ mothers; nine fertile G₀ females bred pure *v*⁺ progenies as a result of the development of some of the *v*⁺ injected female germ cells inside otherwise germline-less *v* females. The progeny of mosaic G₀ females were submitted to a PCR analysis designed to detect the *gyp111fs3* tagged *gypsy* element (see Materials and methods).

The rationale and results of the PCR analysis are presented in Table II. The two mosaic G₀ females, R1 and R2, that were obtained in the 3R control experiment behaved as expected: none of their 10 individual G₁ progenies inherited the *gyp111fs3* tagged element present in their restrictive soma. In contrast, the results of Experiment 3P showed that when present in a permissive soma, this element is frequently transmitted to the progeny of the naive restrictive OR(R) germline: each of the seven mosaic females, P1-7, gave at least one positive G₁ progeny. The amount of the PCR product was much lower than with positive PCR controls performed with single

EXPT. 3P

Fig. 3. An assay to monitor the invasion of a restrictive female germline by *gypsy* proviruses coming from a permissive soma. In the first generation of the experiment (G₀), mosaic females were obtained by transplantation of germ cells (donor) into a mixed population of permissive and restrictive embryos (recipient). To perform the control experiment (Experiment 3R), the progeny of the *v flam*^{FM7c}/*ovo*^{D1} *v flam*^{ovoD1} restrictive embryos was treated in exactly the same way as in Experiment 3P. Except for the *v*⁺ *flam*^{OR(R)} donor, all the genotypes were rich in functional *gypsy* proviruses. The genotype of the restrictive FM7c balancer chromosome of the A67 stock (see Materials and methods) is indicated in boldface. The males used for the G₀ test-cross originated from an isolate of the permissive MG stock in which the *flam*^I permissive allele had mutated towards the restrictive allele *flam*^{IR} (Pelisson et al., 1997). *gyp111fs3* is a tagged provirus which can be detected by a specific PCR test (see text and Materials and methods). Genotypes are not fully represented, only the markers useful for the experiment are indicated.

flies containing one copy of *gyp111fs3* per cell (data not shown). This suggests that the tagged *gypsy* had not integrated into the gametes of the G₀ female. Circumstantial evidence for its subsequent integration was provided by the heritability of the positive signals across two further generations. The DNA of samples of 50 G₂ flies from G₁ pairs containing at least one positive parent were mass-extracted and submitted to the *gyp111fs3* PCR test. Only two out of 10 such tests were negative. Moreover, PCR was also positive for one out of 11 individual sib-matings set up in the G₂ progeny of three double positive G₁ pairs (P1c-P1c, P1d-P1d and P4a-P1f). The *gyp111fs3* DNA detected in this G₂ positive pair was itself transmitted one generation further, since its two samples of 50 G₃

Table II. Transmission of the somatic *gyp111fs3* element to the progeny of mosaic females

Experiment	G ₁ flies		50 G ₂ flies	G ₂ pairs
3R	R1a ♀	—	—	ND
	R1a ♂	—		
	R1b ♀	—		
	R1b ♂	—		
	R1c ♂	—		
	R1d ♂	—		
	R1e ♂	—		
	R1f ♂	—		
	R2a ♀	—		
R2a ♂	—			
3P	P1a ♀	ND	+	ND
	P1a ♂	+		
	P1b ♀	ND	+	ND
	P1b ♂	+		
	P1c ♀	+	+	0/4
	P1c ♂	+		
	P1d ♀	+	ND	1/3
	P1d ♂	+		
	P1e ♀	+	+	ND
	P1e ♂	ND		
	P3a ♀	+	+	ND
	P2a ♂	+		
	P4a ♀	+	+	0/4
	P1f ♂	+		
	P4b ♀	+	+	ND
	P4b ♂	ND		
	P5d ♀	+	—	ND
	P5d ♂	—		
	P6b ♀	+	—	ND
	P6b ♂	ND		
	P7b ♀	+	+	ND
	P7b ♂	ND		
	P5a ♂	w		
	P5c ♀	—		
	P5c ♂	—		
	P6a ♂	w		
	P6c ♀	+		
	P6c ♂	—		
	P7a ♂	—		

Some of the G₁ progeny of mosaic females were sib-mated to form the indicated G₁ pairs; their DNA was then extracted individually and tested for the production of the 0.5 kb *gyp111fs3*-specific PCR band (–, no band; +, strong band; w, weak band; ND, not determined). The progeny of the individual G₁ crosses was then treated in the same way, except that mass DNA extractions were performed (either samples of 50 G₂ flies or mixtures of both parents of a G₂ pair mating). In the latter case, results are expressed as the number of positive pairs over the total number of G₂ pairs tested.

progenies were both PCR positive (data not shown). These results indicate that the increase of *gypsy* copy number can result from expression in the permissive female soma, transfer to the germline (irrespective of the genotype and *gypsy* content of this tissue) and subsequent integration into some germ-cells of the progeny.

Fig. 4. Rationale of the assay to monitor mobilization of the *gyp111fs3* tagged provirus. The transmission of the transgene carrying *gyp111fs3* can be followed by the colored eye phenotype due to the *w*⁺ transgenesis marker. Production of the 0.5 kb *gyp111fs3*-specific PCR band from the DNA of transgene-free G₁ flies (i.e. white-eyed individuals) discloses transposition of this tagged provirus away from the transgene.

Evidence for *env*-independent mobilization of an *env*-defective *gypsy* element

To study whether the Env products are necessary for mobilization, we tagged a *gypsy* element by insertion of a short sequence into the *env* gene, which allows detection of this element by PCR. As shown in Figure 1B, the resulting *gyp111fs3* element cannot produce any functional Env since, because of the frameshift induced by the tag, only the N-terminal half of the surface subunit is coding. If Env products are required for *gypsy* mobilization, this element should not be mobilized in the absence of any Env-producing helper element. It was introduced by P element-mediated transformation in the permissive *wOR(P)* stock. In order to prevent its mobilization inside the transgenic stocks, the transgenes were maintained through paternal lineage by discarding transgenic females at every generation and backcrossing heterozygous transgenic males with females of the *wOR(P)* stock, as described in the Materials and methods and Figure 4. The behaviour of the tagged *gyp111fs3* element was studied by performing a PCR transposition assay. In this assay, *w*⁺ females of the transgenic stocks, which were therefore heterozygous for the transgene and homozygous for the *flam*^{OR(P)} permissive allele (see G₀ females in Figure 4), were mated with males of the *wOR(P)* stock, and the G₁ progeny which inherited the tagged transgenic element were discarded by selecting against the *w*⁺ transgenesis marker. The PCR test described above was then used to look for the presence of putative tagged DNA copies in these transgene-free *w* G₁ flies after they had been allowed to breed the next generation (G₂), either by sib-mating or by outcrossing with the isogenic *wOR(P)* stock. This PCR was performed with samples of 20–25 *w* G₁ flies for each experiment, and with either individual G₂ flies or samples of 50 flies for some G₂ progenies. This experiment was performed with three independent transgenic insertions of the *gyp111fs3* construct, namely the transgene #50 already used in the above experiment, as well as transgenes #59 and #47 (Table III, Experiments 4.50, 4.59 and 6, respectively).

A clear *gyp111fs3*-specific PCR product was obtained with the DNA extracted from each of the nine samples of

Table III. Env-independent mobilization of tagged proviruses

Experiment	Genotype of G ₀ ♀	Transgene	G ₁		G ₂	
			Flies tested by PCR	Positive flies Total	Flies tested by PCR	Positive flies Total
4.50	<i>gyp111fs3^a/+</i>	# 50 ^a	1 (25 w ^{OR(P)} ♀ × 25 w ♂)	1/1	(25 ♀ × 25 ♂)	1/1
			1 (25 w ♀ × 25 w ^{OR(P)} ♂)	1/1	6 (1 ♀ × 1 ♂)	2 ♂/12 = 20%
					(25 ♀ × 25 ♂)	1/1
4.59	<i>gyp111fs3^a/+</i>	# 59 ^a	1 (25 w ^{OR(P)} ♀ × 25 w ♂)	1/1	(25 ♀ × 25 ♂)	1/1
			1 (25 w ♀ × 25 w ^{OR(P)} ♂)	1/1	6 (1 ♀ × 1 ♂)	0/12
					(25 ♀ × 25 ♂)	0/1
5	<i>gyp6Kfs3^a/+; gyp111fs3/+</i>	# 47	35 w ♂	10/35 = 29%		
		# 213	33 w ♂	7/33 = 21%		
		# 247	35 w ♂	4/35 = 11%		
		TOTAL		21/103 = 20%		
5E	<i>gyp6Kfs3^a/+; gyp111/+</i>	# 11	32 w ♂	6/32 = 19%		
		# 13	30 w ♂	6/30 = 20%		
		# 75	35 w ♂	7/35 = 20%		
		TOTAL		19/97 = 20%		
6	<i>gyp111fs3^a/+</i>	# 47 ^a	5 (10 w ♀ × 10 w ♂)	5/5	5 (20 ♂)	7/100 = 7%
6A	<i>gyp111fs3^a/+; CAct-env/+</i>	# 47 ^a	5 (10 w ♀ × 10 w ♂)	5/5	5 (~20 ♂)	16/101 = 16%
6G	<i>gyp111fs3^a/+; gyp6Kfs1/+</i>	# 47 ^a	5 (10 w ♀ × 10 w ♂)	5/5	5 (~20 ♂)	6/101 = 6%

All the flies in these experiments were either homo- or hemizygous for the *w flam^{OR(P)}* permissive X chromosome.

^a indicates which of the *env*-defective constructs, present in the genome of G₀ females, was PCR-tested in their transgene-free G₁ and G₂ progeny. Experiments denoted with the same numbers were done simultaneously. In Experiments 4.50 and 4.59, 25 white-eyed G₁ progenies were mass-mated in both directions with flies of the isogenic w^{OR(P)} stock; in Experiment 6, they were sib-mated. In Experiments 5 and 5E, individual G₁ males were PCR-tested without mating. PCR results are given as the ratio and/or the percentage of positive tests. In the progeny of each G₁ mass-mating, G₂ flies were either collectively or individually tested.

G₁ flies studied, i.e. two samples of 25 females or males in Experiment 4.50 and 4.59, and five samples of 10 males and 10 females in Experiment 6. Since these G₁ flies had been mated before the test, it was possible to study the genetic transmission of this positive PCR signal and make sure that it did not result from trivial DNA contamination originating either from the mothers or from the w⁺ siblings. In Experiments 4.50 and 4.59, the positive PCR signal observed in one of the two samples of 25 G₁ females was indeed transmitted to a sample of 50 G₂ progenies. That this probably does not result from maternal transmission of a cytoplasmic *gyp111fs3* DNA was shown by the paternal transmission of both G₁ males PCR signals to samples of 50 G₂ flies. Moreover, out of 12 individual G₂ progenies analysed in Experiment 4.50, two gave a positive PCR signal, which gives an estimated frequency of male transmission similar to 10⁻¹. The same order of magnitude was obtained in a more accurate measurement of the frequency of global transmission by both G₁ males and females in Experiment 6, since as many as seven of the 100 G₂ sons of five samples of 20 G₁ flies inherited the positive signal from their mother and/or father. In Experiment 4.59, the minimal estimation of this frequency is one order of magnitude lower if one assumes that a single G₂ individual was responsible for the positive signal observed in one sample out of a total of 112 G₂ flies tested (two samples of 50 flies and 12 individual tests). Finally, to ensure that the *gyp111fs3* DNA had actually inserted into the genomic DNA, the meiotic segregation of a G₂ positive signal was studied. In Experiment 4.50,

23 G₃ flies of one of the two positive G₂ males crossed with a negative sister were individually PCR tested. As shown in Figure 5, nine of these flies produced the *gyp111fs3*-specific PCR product, which is not statistically different from the expected 1:1 monofactorial segregation ($\chi^2 < \chi^2_{0.05}$).

One may conclude from this analysis that the signals obtained by PCR in Experiments 4.50 and 6 resulted from the insertion of the *gyp111fs3* provirus into the genome of ~10% of the germ cells of G₁ w flies and its subsequent Mendelian transmission through male and female meiosis. The lower transmission efficiency observed in Experiment 4.59 might result from position effect possibly reducing the level of expression of transgene #59 in the G₀ females, which would in turn result in a lower frequency of transposition.

To make sure that the autonomous mobilization of the *env*-defective *gyp111fs3* element is actually independent of the *gypsy* envelope, we checked that no Env products could be detected in the permissive females carrying this element. Immunostaining of sectioned *gypsy*-rich permissive females had previously shown that the production of Env is restricted to the follicle cells of the ovaries (data not shown). A 1:1 mixture of the 7B3 and 8E7 anti-Env monoclonal antibodies (mAbs) was incubated with whole mount ovaries. Ovaries of the transgenic line #47 (Figure 6B), which contains one copy of the *gyp111fs3* *env*-defective element, did not disclose but the very low level of background signal seen in the w^{OR(P)} strain used for transgenesis (Figure 6A). In contrast, a positive control

Fig. 5. Genetic evidence for an *env*-independent genomic integration of the *gyp111fs3* *env*-defective provirus. The *gyp111fs3*-specific PCR products obtained with the progeny of one positive G_2 male in Experiment 4.50 (see Table III) are shown. The genomic DNAs from 23 G_3 flies were used individually as templates for PCR experiments using the 5423+ and L1- primers described in Materials and methods and Figure 1B.

signal was indeed obtained with transgenic females containing one copy of the functional *gyp111* element (see below and Figure 6C).

All these results indicate that the *gypsy* endogenous retrovirus can be mobilized in the absence of any immunologically detectable *gypsy* Env in the follicle cells. We then tested the possibility that Env proteins might further increase this mobilization.

Production of the *gypsy* Env in follicle cells does not increase the mobilization of *env*-defective *gypsy* elements

Env produced by *gyp111*. This is the *gypsy* element used to construct the defective *gyp111fs3* tagged element. It originates from the *pDm111* clone (Bayev *et al.*, 1984) and has been shown to be active by its ability to transpose autonomously after introduction by transgenesis into the genome of the permissive *SS* strain which is devoid of functional *gypsy* proviruses (Kim *et al.*, 1994b). Three transgenic lines containing this element (#11, #13 and #75) were obtained in the empty *wOR(P)* background and the transgenes were stably maintained through patroclinous lineage, as explained above and in the Materials and methods. Each of these transgenes was used to complement *gyp6Kfs3*, another *env*-defective element described in Materials and methods and Figure 1B. An experimental scheme similar to the forthcoming was used to detect the non-Mendelian transmission of this tagged *gypsy* to 30–35 transgene-free sons of *w flam^{OR(P)}/w flam^{OR(P)}; gyp111/+; gyp6Kfs3/+* females (Table III, Experiments 5E). In the control experiments (Table III, Experiments 5), any one of three *env*-defective *gyp111fs3* transgenes (#47, #213 and #247) were used as helpers instead. The fact that *gyp6Kfs3* had got a different tag from that of *gyp111fs3* enabled its mobilization to be followed independently of that of the other tagged *gypsy* present in the control females of Experiment 5. For that purpose, a PCR assay similar to the one described above but using the *gyp6Kfs3*-specific L5938/*flag* primer was performed to look for the presence of copies of this element in individual $w G_1$ males. As illustrated in Figure 6C for transgene #75, each of the three *gyp111* transgenes produced detectable amounts of Env proteins in the follicle cells of transgenic females. About the same signal was observed in G_0 females of Experiment 5E (data not shown). In contrast, no significant signal was noticed in the *w flam^{OR(P)}/w flam^{OR(P)}; gyp6Kfs3/+; gyp111fs3/+* control females of Experiment 5 (data not shown). As shown in Table III, ~20% of the transgene-free G_1 males inherited the *gyp6Kfs3* element whether it had been associated in their mothers with the *gyp111* Env-producer helper (Experiment 5E) or the *gyp111fs3* *env*-defective element (Experiment 5).

Fig. 6. Expression of *gypsy* Env proteins in the ovaries of various transgenic and control females. Immunofluorescence analysis of young whole mount ovaries using a mixture of both 7B3 and 8E7 monoclonal anti-Env antibodies. The following genotypes were studied: (A) *w flam^{OR(P)}/w flam^{OR(P)}*; the females of the *wOR(P)* strain, which does not contain any functional *gypsy* provirus and in which the transgenic strains were obtained, display a very low background level of fluorescence; (B) *w flam^{OR(P)}/w flam^{OR(P)}; gyp111fs3/+*; ovaries of transgenic line #47 showing the same background level of fluorescence; (C) *w flam^{OR(P)}/w flam^{OR(P)}; gyp111/+*; transgene #75 is given as an example of the clear Env signal observed in the monolayer of follicle cells surrounding the oocyte at stage 10; (D) and (E) have the same genotypes as in (B) except for the presence of the *gyp6Kfs1*(#79) and *CAct-env*(#6) additional transgenes, respectively, which results in an Env signal similar to that observed in (C).

Env produced by gyp6K. To rule out the possibility that the absence of any Env effect on the frequency of *gypsy* mobilization was due to mutation of the *gyp111 env* gene, the Env of another *gypsy*, *gyp6K*, was also produced in similar experiments. Two constructs were made to produce it under the control of two different promoters; either the *gypsy* promoter of the *gyp6Kfs1* transgene or the *actin5C* heterologous promoter (Figure 1C and A, respectively). The product of the *env* gene from *gyp6K* had been shown previously to be functional by its ability to make a Moloney-based vector infect *Drosophila* S2 culture cells (Teyssset *et al.*, 1998). Both promoters were checked to drive correct expression of the Env products as shown in Figure 6D–E. The *gyp111fs3* PCR transposition assay described above was performed with single flies in the G₂ progeny of *w flam^{OR(P)}/w flam^{OR(P)};gyp111fs3/+* (Experiment 6), *w flam^{OR(P)}/w flam^{OR(P)};gyp111fs3/+;gyp6Kfs1/+* (Experiment 6G) and *w flam^{OR(P)}/w flam^{OR(P)};gyp111fs3/+;CAct-env/+* (Experiment 6A) G₀ females. As shown in Table III (Experiment 6), the *env*-defective *gyp111fs3* element was found inserted in 7% of G₂ flies. This frequency was not significantly increased by the *gyp6K* Env protein, whether its expression in G₀ females was driven by the homologous *gyp6K* (Experiment 6G, $p = 0.99$) or the heterologous *actin 5C* (Experiment 6A, $p = 0.08$) promoter. In another experiment, it was also observed that the frequency of insertion into the *ovo* gene of the *gypsy* elements provided by the OR(721) stock did not increase in the presence of the transgene *Cact-env* (data not shown).

Discussion

The results presented in the first part of this paper explain the apparent discrepancy between the maternal effect of *flamenco* on *gypsy* transposition and the somatic specificity of the regulation *flamenco* exerts on *gypsy* expression. They indeed show that: (i) in order to transpose, active *gypsy* proviruses must be present in the permissive mothers of the flies in which new *gypsy* insertions occur; (ii) the regulation of this process is not germline dependent and even when inherited from the mother, active proviruses are not mobilized if they were only present in a permissive female germline clone surrounded by a restrictive soma; (iii) new *gypsy* insertions even occur in the flies derived from restrictive germ cells devoid of *gypsy* proviruses, provided these germ cells were present in a *gypsy*-containing permissive soma. These results show that insertion of new proviruses in the germline requires the expression of *gypsy* in the soma of the females in the progeny of which it occurs. They eventually provide direct evidence for the previous insight that transposition would involve the transfer of the element from somatic to germ cells (Pelisson *et al.*, 1994).

Therefore, *gypsy* clearly relies on somatic expression for the increase of its copy number inside the germline. This peculiar strategy of replication is reminiscent of the recurrent acquisition by SWR/J mice of new germline ecotropic MuLV proviruses by virtue of retroviral infection by proviruses expressed in somatic tissues of the female reproductive apparatus (Lock *et al.*, 1988). By analogy, the highly specific somatic expression of the 412 and ZAM *Drosophila melanogaster* retroelements (in the embryonic

gonad and the adult ovary, respectively) could be tentatively interpreted as a possible way for these elements to gain access to the germinal cells through the production of hypothetical infectious particles (Brookman *et al.*, 1992; Leblanc, 1998). Concerning the rat OST retrovirus-like sequences (Godwin *et al.*, 1995), we also do not know how relevant their specific transcription in the somatic tissues adjacent to follicles is to their replication. Similarly, the biological significance of the mass production of endogenous retroviral particles and antigens in the placenta of animals and humans is still a matter of speculation (Villareal, 1997; Harris, 1998).

The data presented in this paper suggest that the transmission of *gypsy* from the soma to the germline does not require expression of the *env* gene. In fact, *env*-defective *gypsy* elements insert themselves at high frequency in the germline of flies derived from permissive females devoid of any transcomplementing active *gypsy* provirus. Moreover, the frequency of new insertions was not further increased when the *gypsy* Env protein was produced by an *env* transgene under control of either the ubiquitous *actin* or the tissue-specific *gypsy* promoter. One, at least, of the two *env* genes used in these complementation experiments is known to be functional since it had previously disclosed the ability to pseudotype a Moloney-based retroviral vector and make it infect *Drosophila* cultured cells (Teyssset *et al.*, 1998). These observations do not fit with the hypothesis of the germline infection that was previously proposed to explain the maternal effect of the *flamenco* gene on *gypsy* transposition (Pelisson *et al.*, 1994; Song *et al.*, 1997). They rather suggest some non-infectious pathway for the somatowards-germen *gypsy* transfer. Non-enveloped particles might, for instance, enter the oocyte by endocytosis as cytochemical tracers do (Giorgi and Jacob, 1977; Giorgi, 1980). However, a previous electron microscopy study failed to detect such particles in the yolk spheres, whereas they were seen to accumulate inside the follicle cells (Lecher *et al.*, 1997). The hypothesis of an infection process cannot be completely ruled out if, for instance, some endogenous Env, not reacting with the two monoclonal antibodies used in this study, is assumed to be produced by the wOR(P) stock in non-limiting amounts. For such a hypothesis, amplification of this endogenous retrovirus in the germline would involve germline infection by the exogenous phase of a virus life cycle, which would take advantage of the helper activity of such a putative endogenous *env* host gene.

These data leave quite open the question of the function of the viral *env* gene if it is not required for completion of the endogenous replication cycle. There is still controversy about the adaptative function the *env* genes of some vertebrate ERVs might have for their host, either in protection against superinfection (Gardner *et al.*, 1991; Villareal, 1997) or in placental development (Venables *et al.*, 1995; de Parseval and Heidmann, 1998). Moreover, no functional Env seems to be encoded by *gypsy* in other *Drosophila* species: none of the few *gypsy* clones isolated so far from *Drosophila subobscura* (Alberola *et al.*, 1997) and *Drosophila virilis* (Mizrokhi and Mazo, 1991) have a functional *env* gene. Therefore, in *Drosophila melanogaster*, *gypsy* itself might be under selective pressure to keep its own *env* gene functional. This would be

the case if *gypsy* relies on horizontal transfer for its maintenance in the genome of natural populations. Although not yet thoroughly studied, natural populations contain restrictive *flamenco* alleles (Pelisson *et al.*, 1997; A.Kim, N.Prud'homme and A.Bucheton, unpublished data) and the *env*-independent endogenous replication process described here appears to be strongly repressed in restrictive genotypes. If the replication level is too low to compensate for extinction by mutational inactivation, infection might be the only way to recover a minimal number of active proviruses. *Gypsy* would thus be able to lead a double life in *Drosophila melanogaster*: on one hand, it would require the Env products to settle in new hosts by horizontal transfer, but on the other hand, the family of vertically transmitted endogenous proviruses would be able to amplify owing to the *env*-independent soma-towards-germen transfer described here.

Materials and methods

Plasmid construction

pgyp6Kfs3 and *pgyp11fs3* were obtained by ligation of two different oligonucleotides, CTAGTAGACTACAAGACCACGATGACAAG and CTAGCTTGTATCGTGCCTCGAC, respectively, with the *SpeI*-generated staggered cuts at position 5926 into the p6K (Lyubomirskaya *et al.*, 1990) and pDm111 (Bayev *et al.*, 1984) *gypsy* clones. *pgyp6Kfs3* resulted from the end-filling with Klenow of the *NcoI* site of p6K at position 1201. The four *BamHI* fragments containing each of these three constructs and the wild-type *gyp11* element were each inserted into the *BamHI* site of the pW6 transformation vector (Klemenzen *et al.*, 1987) in the opposite orientation to that of the *white* transformation marker.

pCACT-env was constructed by inserting the p6K *env*-containing *BamHI* fragment of *Act-env* (Chalvet *et al.*, 1998) into the *BamHI* site of the *pCaSpeR-act* transformation vector (Thummel *et al.*, 1988) in the proper orientation to put it under control of the *actin* 5C promoter.

The final clones were sequenced as described previously (Sanger *et al.*, 1977) to ensure that they had actually incorporated the expected modified sequences as represented in Figure 1.

Drosophila strains and genetic crosses

The strains were maintained on standard *Drosophila* medium (Gans *et al.*, 1975). All experiments were carried out at 25°C. All crosses, including the mass matings of 25 pairs of flies were performed in vials. Genetic symbols are as described previously (Lindsley and Zimm, 1992). The *ovo*^{D1} inactivation assay was described earlier (Kim *et al.*, 1994a; Prud'homme *et al.*, 1995). To induce somatic recombination, early second instar larvae were irradiated with 1000 R X-rays as described previously (Perrimon and Gans, 1983). The genotype of the resulting germinal clones were determined by progeny testing. Germ cell transplantation was performed as described (Sanchez and Santamaria, 1997).

Stocks devoid of functional gypsy proviruses. The permissive OR(P) and the restrictive OR(R) stocks are two sublines of the wild-type Oregon R stock. As reported previously (Pelisson *et al.*, 1997), OR(P) is one of the stable permissive strains, suggesting that it is devoid of active *gypsy* copies. Another empty permissive stock, wOR(P), was obtained by recombination of the *w¹¹¹⁸* genetic marker with the *flam*^{OR(P)} allele of the OR(P) stock. Although only its autosomes were tested for the lack of any active *gypsy*, OR(R) is also very likely to be an empty stock because of its common origin with OR(P).

Gypsy-rich stocks. The *ovo*^{D1} *v flam*^{ovoD1} stock used here contains functional *gypsy* proviruses on the autosomes. The MG stock was described previously (Prud'homme *et al.*, 1995; Pelisson *et al.*, 1997). It contains the permissive *flam*¹ allele. The *flam*^{OR(P)} allele of the permissive OR(P) strain was recombined into the genetic background of two *gypsy*-rich sublines of the MG stock. The two resulting *gypsy*-rich permissive recombinant chromosomes were maintained in balanced stocks, OR(520)/FM3 and OR(721)/FM3, where *gypsy* mobilization was prevented by the FM3 dominant restrictive balancer, as described previously (Pelisson *et al.*, 1994). The *flam*^{2G} permissive allele was extracted by recombination from the *Df(1)B12* chromosome, which means that, in contrast to the conclusion drawn from previous experiments

(Prud'homme *et al.*, 1995), the B12 deficiency carried by this chromosome does not affect the *flamenco* locus. The active *gypsy* proviruses carried by the distal part of the X chromosome from the MG stock were then associated with this new permissive allele in a *y v f mal flam*^{2G} recombinant chromosome. Finally, the second chromosome carrying the *gyp11fs3* transgene #50 (see below) was combined with this X chromosome to make the balanced *gypsy*-rich stock A67.

Transformed lines. All transgenes were recovered as coloured-eyed *w⁺* individuals after P-mediated transformation into the wOR(P) permissive empty stock (Spradling, 1986). To prevent any risk of mobilization inside transgenic stocks, heterozygous transgenes were maintained through paternal lineage by discarding females carrying the transgene at every generation and backcrossing males with either *w* sisters or with females of the wOR(P) stock (see Figure 4). The transformed lines were examined by Southern blot hybridization to check for the integrity of the transgene and the number of copies present. The *gyp6Kfs3* line #79 and the *CACT-env* line #6 were chosen because of the location of their transgene on chromosome 3 (data not shown) and their ability to produce easily detectable amounts of the Env protein by immunofluorescence in follicle cells (see Figure 6D and E).

Ovo^{D1} inactivation assay

The *ovo* gene is located on the X chromosome at cytological position 4E2. It is a hot spot of insertion of *gypsy* (Mevel-Ninio *et al.*, 1989). Crosses of permissive females containing active *gypsy* proviruses with males carrying the *ovo*^{D1} female dominant mutation produce fertile daughters at high frequency. This results from insertions of *gypsy* into the *ovo* gene carried by the paternal chromosome. To estimate the frequency of these insertions, the daughters are scored for zero, one or two ovaries. In most cases, fertile females have one ovary, indicating that most integrations occur late in embryogenesis after colonization of the gonads by germ cells. The frequency of females with two ovaries is approximately the square of that of females with one ovary, suggesting that they result from two independent events. Such females are therefore counted twice to calculate the frequencies of reversion of *ovo*^{D1}. Fertile daughters also may result from mitotic crossing-over, producing germ cells devoid of the *ovo*^{D1} allele. These events can be detected by studying segregation of the markers in the progeny. Their frequency is very low (<0.5%).

Allelism test of the flam allele carried by the ovo^{D1} chromosome

The status of a given *flamenco* genotype is determined according to the ability of females carrying this genotype to allow *gypsy* insertion in their progeny. Testing the *flam*^{ovoD1} allele associated with the dominant sterile *ovo*^{D1} mutation requires transmission of this chromosome through a female germline, which is only possible if *ovo*^{D1} first undergoes a recessive null *ovo*^o mutation (Busson *et al.*, 1983). This happens in as many as 10% of the female progeny of MG females, which can then be tested by the *ovo*^{D1} inactivation assay (see above). Essentially, these *ovo*^o *v flam*^{ovoD1} *y v f mal flam*¹ fertile F₁ females were further backcrossed to *ovo*^{D1} *v* males and the proportion of fertile F₂ female progeny was scored. The percentage was then doubled to compensate for the inability of half of the F₂ female progeny to become fertile by mutation of *ovo*^{D1} towards *ovo*^o (*ovo*^o/*ovo*^o mutants are as sterile as their *ovo*^o/*ovo*^{D1} non-mutant siblings). Given the low level of *ovo*^{D1} reversion (Table I), we considered *flam*^{ovoD1} as weakly restrictive and dominant over *flam*¹. However, its combination with the *flam*^{2G} permissive allele led to high levels of *ovo*^{D1} reversion, suggesting that this restrictive allele may be either dominant or recessive depending on the permissive allele considered.

gyp11fs3 and gyp6Kfs3 PCR assays

Fly DNA was extracted as follows. Individual frozen adult flies were homogenized in 100 µl of buffer (0.1 M Tris-HCl pH 9.0, 0.1 M EDTA, 1% SDS) with plastic pestles in Eppendorf tubes. Each homogenate was incubated for 30 min at 70°C, and then 14 µl of 8 M potassium acetate was added and left on ice for 30 min. After spinning for 15 min at 4°C, the supernatant was transferred carefully to a fresh Eppendorf tube. DNA was then precipitated by adding a 0.5 vol of isopropanol at room temperature and spun down for 5 min. The DNA pellet was washed carefully with 70% ethanol, respun, dried and resuspended in 10 µl of water. For mass extractions the same procedure was scaled up to samples of 50 flies by increasing volumes up to five times, except that DNA was resuspended in 100 µl. From 50 ng (corresponding to 1/10 of an individual extraction) to 300 ng (corresponding approximately to half a

fly for mass extractions) of DNA was used as template for PCR. PCR was done as follows: one denaturation cycle at 95°C for 5 min, then 95°C for 45 s, 65°C for 45 s, 72°C for 30 s for 30 cycles. *Taq* DNA polymerase (2.5 U) in 50 µl one times buffer A (Promega) was used with 200 µM dXTPs, 2.5 mM MgCl₂ and 0.4 µM of each primer. As a positive control for the presence of similar amounts of template DNA, the reliability of the PCR results was checked by performing a ribosomal DNA amplification in every case (data not shown).

The oligonucleotide 5423+, 5'-TCTGTTCTTATTAAGGGGAGGG-TGG-3' was combined to either of the two following primers, specific of *gyp111fs3* and *gyp6Kfs3*, respectively: L1-, 5'-CTAGGTCGACGACGATGACAAG-3'; and L5938*flag*, 5'-CTAGCTTGTCATCGTCGT-CCTTG-3' (Figure 1B).

Whole-mount immunocytochemistry

Two anti-gypsy-Env monoclonal mouse antibodies (MAbs), clones 7B3 and 8E7 (Song et al., 1994), were generously supplied by V.G.Corces (The Johns Hopkins University, Baltimore, MD). Ovaries were dissected from well-fed 3- to 5-day-old females and fixed in 1× phosphate-buffered saline (PBS)/Tween 20 0.1%/Triton 1%/formaldehyde 2%/0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF) for 30 min at room temperature. The fixative was removed by rinsing three times with PBT (1× PBS/0.5% bovine serum albumin (BSA)/Triton) and ovaries were blocked for 1 h at room temperature in PBT complemented with 5% preimmune horse antibodies (horse Ab). The primary antibody, an equal mixture of the two anti-gypsy-Env MAbs, was applied as a 2:5 dilution (1:5 each) in PBT-horse Ab, and incubated with ovaries overnight at 4°C. After three changes of PBT in 60 min, ovaries were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (Vector Laboratories) diluted at 1/100. Ovaries were then washed with three changes of PBT for 1 h at room temperature. Ovaries were mounted in Vectashield medium (Vector Laboratories) and observed by fluorescence microscopy on a Leica MRD microscope.

Statistical analyses

Statview Student (Abacus Concepts) was used to calculate *p* values by a contingency table analysis. The reported values are continuity-corrected *p* values.

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