The *flamenco* Locus Controls the *gypsy* and *ZAM* Retroviruses and Is Required for Drosophila Oogenesis

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ABSTRACT

In Drosophila, the as yet uncloned heterochromatic locus *flamenco* (*flam*) controls mobilization of the endogenous retrovirus *gypsy* through the repeat-associated small interfering (rasi) RNA silencing pathway. Restrictive alleles (*flam^R*) downregulate accumulation of *gypsy* transcripts in the somatic follicular epithelium of the ovary. In contrast, permissive alleles (*flam^P*) are unable to repress *gypsy*. *DIP1*, the closest transcription unit to a *flam*-insertional mutation, was considered as a good candidate to be a *gypsy* regulator, since it encodes a dsRNA-binding protein. To further characterize the locus we analyzed *P*-induced *flam* mutants and generated new mutations by transposon mobilization. We show that *flam* is required somatically for morphogenesis of the follicular epithelium, the tissue where *gypsy* is repressed. This developmental activity is necessary to control *gypsy* and another retroelement, *ZAM*. We also show that *flam* is not *DIP1*, as none of the new permissive mutants affect the *DIP1* coding sequence. In addition, two deletions removing *DIP1* coding sequences do not affect any of the *flamenco* functions. Our results suggest that *flamenco* extends proximally to *DIP1*, spanning >130 kb of transposon-rich heterochromatin. We propose a model explaining the multiple functions of this large heterochromatic locus.

TRANSPOSABLE elements constitute an important part of eukaryotic genomes. Among them retrotransposons, which replicate by transcription of an RNA intermediate, subsequent reverse transcription, and insertion of new copies elsewhere in the genome, make up at least 45% of the human genome (LANDER *et al.* 2001). Mobilization of retrotransposons is tightly controlled so as not to be overly deleterious. Drosophila offers an unique system to study interactions between these parasite-like elements and their host given the wealth of genetic tools available in this organism. In Drosophila, retroelements are silent most of the time. However, several strains are known in which massive mobilization of one or several elements occurs or has occurred (MÉVEL-NINIO *et al.* 1989; PASYUKOVA *et al.* 1997; DESSET *et al.* 2003).

For the past decade our laboratory has been engaged in studying the control of the mobilization of *gypsy*, an endogenous retrovirus of *Drosophila melanogaster* (BUCHETON 1995; PRUD'HOMME *et al.* 1995). Endogenous retroviruses can be considered as a particular class of long terminal repeat (LTR)-containing elements. They are inserted in the genome of their host and therefore transmitted through the germline. Some of them, including

gypsy, can produce infectious particles and consequently infect horizontally new individuals (KIM *et al.* 1994a; SONG *et al.* 1994). The genetic organization and replication cycle of *gypsy* is similar to that of vertebrate retroviruses (MARLOR *et al.* 1986; AVEDISOV and ILYIN 1994; PELISSON *et al.* 1994).

D. melanogaster strains generally contain very few copies of active *gypsy* located in euchromatin. Genetic analysis of *gypsy* proviral amplification has shown that it is controlled by an as-yet-uncloned locus, *flamenco* (*flam*), located in the centromeric heterochromatin of the *X* chromosome (PRUD'HOMME *et al.* 1995; ROBERT *et al.* 2001). Two classes of alleles, restrictive and permissive, are present in natural populations in about the same proportions (PELISSON *et al.* 1997). Restrictive alleles (*flam^R*) are dominant and repress mobilization of functional *gypsy* proviruses. Therefore, strains containing *flam^R* alleles are stable with regard to *gypsy* copy number. Conversely, permissive alleles (*flam^P*) allow a high level of *gypsy* replicative transposition in homozygous strains (PRUD'HOMME *et al.* 1995). However, most of the permissive stocks are stable because they do not contain functional *gypsy* proviruses (KIM *et al.* 1994b; PELISSON *et al.* 1997). In homozygous permissive females, the transcripts of active *gypsy* proviruses and virus-like particles accumulate in the somatic follicular epithelium (FE) surrounding germinal cells of the ovary (PELISSON *et al.* 1994; LECHER *et al.* 1997). It has been shown that restrictive

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flam^R alleles repress *gypsy* transcript accumulation through a novel repeat-associated small interfering (rasi) RNA pathway, which is *piwi*-dependent (SAROT *et al.* 2004; PELISSON *et al.* 2007), similar to the recently described pathway, which controls repetitive sequences in Drosophila (SAITO *et al.* 2006; VAGIN *et al.* 2006).

When this study began, one unique *flam* mutant [*flam^{by+(P)}*] was known. It was generated by insertion of the *P{lyB}* transposon into the 20A region of the Drosophila genome (ROBERT *et al.* 2001). Genetic and molecular studies of *flam* have been hindered because this insertion is located in an area of heterochromatin close to a sequencing gap of unknown size. The genomic DNA extending proximally to the *P{lyB}* insert (toward the centromere) consists mostly of defective repeated elements, especially truncated retroviral sequences. As such, no transcriptional unit corresponding to a single-copy gene was found. Distal to the *P{lyB}* insert, three different transcription units have been characterized. *DIP1*, the closest to the *P{lyB}* insertion site, encodes a nuclear protein containing two double-stranded RNA-binding domains (DESOUSA *et al.* 2003) and therefore may be considered as a good candidate to function as a *gypsy* regulator.

In this study, we attempted to further characterize the *flam* locus by analyzing new insertional mutations and creating new genome rearrangements by transposon mobilization. This study reveals that, besides its function in *gypsy* repression in follicle cells, *flam* also controls FE morphogenesis. Moreover, the induction of *flam* mutants by insertions or deletions not only results in both female sterility and *gypsy* derepression but also relieves the repression of the *ZAM* retroelement. Our data also indicate that *flam* is not *DIP1* but a distinct multifunctional heterochromatic locus that extends proximally to *DIP1* over >130 kb.

MATERIALS AND METHODS

Drosophila strains: Flies were grown at 25° on standard Drosophila medium. The strain wOR(P) carrying a natural permissive *flam* allele has been previously described (SAROT *et al.* 2004). The BG, KG, and EY collections from which the *flam^{BG(P)}*, *flam^{KG(P)}*, and *flam^{EY(R)}* stocks were obtained are described in BELLEN *et al.* (2004). *PEY(R)* is the *y w* parental strain of *flam^{EY(R)}*. *GE89(R)* is a deletion derivative of the insertional mutant *G1261* sold by the Genexel company. *G1261* contains the *EP* transposon (RORTH 1996) inserted at exactly the same position as *EY* in the *flam^{EY(R)}* mutant. The strains of the laboratory collection were previously described, including the sample of deletions *Df(1)l_x* (PRUD'HOMME *et al.* 1995; ROBERT *et al.* 2001). The *ZAM-lacZ* tester strain (DESSET *et al.* 1999) was kindly provided by C. Vaury.

Northern blots: Ovarian *gypsy* rasiRNAs were detected using a low-molecular-weight Northern blotting method and a small sense RNA probe that were described previously (PELISSON *et al.* 2007). The amount and integrity of the RNA were checked both by staining the gels with 2 mg/ml ethidium bromide and by reprobing the unstripped membranes with an end-labeled oligonucleotide complementary to the abundant mir-13b microRNA (5′-ACTCGTCAAAATGGCTGTGATA-3′).

Rescue experiments: Cosmid 7a is issued from the NotBamNot-CoSper genomic library, kindly provided by J. Tamkun. It consists of 35 kb of genomic sequence including the whole *DIP1* sequence and 29 kb extending upstream of the *DIP1* transcription start site (see ROBERT *et al.* 2001). An *AvrII–BciVI* 8-kb fragment of cosmid 7a, potentially able to produce all *DIP1* transcripts, was subcloned in the *HpaI* and *SpeI* sites of the pCaSpeR4 vector to generate transgenic lines.

DIP1-b and *DIP1-c* cDNAs were issued from an ovarian cDNA library (STROUMBAKIS *et al.* 1994) and from the Berkeley Drosophila Genome Project (BDGP) (EST LD14381), respectively. The *NotI–XbaI* digestion fragment of *DIP1-b* and the *NotI–SmaI* digestion fragment of *DIP1-c*, both cloned into the pUAST vector, were subcloned into the *NotI–XbaI* and *NotI–HpaI* sites of the pCaSpeR-HS vector, respectively. The sterility of *flam^{by+(P)}* mutant females is thermosensitive, partial rescue being observed when L3 and pupal stages are raised at 18° (15 adult progeny per female at 18° for 9-day egg laying). For this reason, our attempt to rescue the sterility was performed by heat-shocking transgenic females at these developmental stages for 1 hr/day at 37°.

Ovary DAPI staining: Ovaries were stained with the nuclear dye DAPI for the examination of ovary morphology. They were fixed in 4% formaldehyde, 1× PBS, 1% Triton (PBT) for 10 min and rinsed twice in PBT. Then, they were placed for 5 min in PBT with 1 μg/ml of DAPI (Sigma, St. Louis). After three rinses they were mounted in PBS:glycerol (1:1). Samples were examined by confocal microscopy using a Leica SP2 UV microscope.

Permissivity and instability assays: The reporter constructs for *gypsy* (p#12) or *ZAM* (pZAM-lacZ) expression in ovaries were described in SAROT *et al.* (2004) and DESSET *et al.* (2003), respectively. They consist of the *gypsy* or *ZAM* promoters fused to the *Escherichia coli lacZ* gene. Permissive *w flam^{OR(P)}/w flam^{OR(P)}* females homozygous for the #12 transgene were crossed to males under test. The *flam* restrictive character is semidominant and daughters do not express high levels of β-galactosidase in their FE cells if their father carries a *flam* restrictive allele. In the same way, *X^U/X^U*; *ZAM-LacZ/ZAM-LacZ* females were crossed to males of lines containing various *flam* alleles. Coloration develops in all egg chambers only if the father carries a U allele. Histochemical staining of β-galactosidase was performed as described in SAROT *et al.* (2004).

Germline clonal analysis: To obtain mosaic females with germline cells homozygous for *flam^{BG(P)}*, we induced mitotic crossing over in *w ovo^+ flam^{BG(P)}/ovo^{D1} v flam^+* flies according to PERRIMON and GANS (1983). Early larvae were irradiated with 1000 rad of X radiation from an X-ray source. Adult females were mated to *w flam^{BG(P)}/Y* males and tested for fertility. Confirmation that the female germline clones were *flam^{BG(P)}* was obtained by examining the male progeny for the white marker. Ovaries were fixed, stained for DAPI, and examined.

P-element excision analysis: As shown in Figure 3, mobilization of the *P{EPgy2}* transposon was induced in *flam^{EY(R)}* G1 males that had received the Δ2-3(99B) source of transposase. These males were crossed to *FM0/flam^{BG(P)}* females and independent excision events were isolated in G2 as *FM0/flam^{EY}* females having lost the *w* and/or *y* markers. Viability of the excised alleles as well as their ability to complement *flam^{BG(P)}* sterility was tested in G3. All 130 tested alleles were viable.

Molecular analysis of the excisants: Deletions of the *P{EPgy2}* transposon were analyzed by amplification assays of genomic DNA using the oligonucleotide primers oligo *a* (*DIP1* sense orientation), GAGGAAACGCAAAACTCTTGTGAA, and oligo *b* (*DIP1* antisense orientation), TGAACACTTTCACTTT GTGCGCTGCAA (see Figure 1). Deletions of sequences flanking

the *P{EPgy2}* transposon were tested by amplification of flanking DNA fragments. We used the following pairs of primers: oligo *a* and oligo *c*, TAAAAAACTTAAGTAGGGATGTCAAA AT; and oligo *b* and oligo *d*, TGCAGTACGATTCT TTCTGT GACCTG (see Figure 1). Controls were performed using DNA from the strain *PEY(R)*, which is the *flam^{EY(R)}* parent. Deletions present in *flam^{EY4(R)}* and *flam^{GE89(R)}* were analyzed by sequencing PCR products. Analysis of 6 kb of DNA sequence proximal and distal to the *P{EPgy2}* insertion site was performed by amplification of overlapping DNA sequences using appropriate pairs of oligonucleotide primers (oligo sequences are available upon request). Although the DNA sequence proximal to the *P{EPgy2}* insertion site consists of repeated sequences, the arrangement of the repeated DNA is unique in the genome and a unique amplification product was always obtained. The genomic sequence flanking proximally the deletion in *flam^{EY18(P)}* was determined by sequencing of the inverse PCR product. A detailed protocol is available on the BDGP webpage at http://www.fruitfly.org/about/methods/inverse.pcr.html. Genomic DNA was digested with *Hpa*II and the flanking sequence was amplified with the PlacI CACCCAAGGCTCTGCTCCCACATT and PwhtI GTAACGCTAATCACTCCGAACAGGTCACA primers. Database searches were carried out by BLAST search using the last release database at the National Center for Biotechnology Information (NCBI) and at FlyBase.

RESULTS

P-element-induced *flam* mutants are both female sterile and permissive for *gypsy* expression: Permissive and restrictive *flam* alleles coexist in natural populations (PELISSON *et al.* 1997). Derepression of *gypsy*, in permissive females, occurs in the somatic FE (PELISSON *et al.* 1994). Thus, we wondered whether *flam* also plays a role in FE formation. We first analyzed females from the wOR(P) strain that carry a natural permissive allele. These females are normally fertile and examination of their ovaries, after DAPI staining, did not reveal any defects (data not shown). Accordingly, either *flam* has no function in the FE or the *gypsy* control function can be inactivated independently from a putative ovarian function.

Next, we examined the only available *flam* mutant, *flam^{by+(P)}*, resulting from insertion of the *P{lyB}* transposon into region 20A1-3 of the restrictive *FM7c* balancer chromosome (ROBERT *et al.* 2001). We eliminated the *sn* female-sterile mutation present on *FM7c* by recombination with the *FM7a* balancer chromosome and found that homozygous *flam^{by+(P)}* females did not lay any eggs (Table 1). Subsequently, we checked that the sterility phenotype of *flam^{by+(P)}* females was actually caused by the *P{lyB}* insertion. Thirty excision events of the *P{lyB}* transposon were studied by Southern blot analysis as described in ROBERT *et al.* (2001). In 20 of them, either precise or imprecise excision was associated with simultaneous reversion toward the restrictive character and restoration of fertility, indicating that the *P{lyB}* insertion was responsible for both mutant phenotypes and had taken place in a noncoding region (data not shown). The 10 excisants that remained permissive were still female sterile. This further strengthened the link

TABLE 1

Complementation tests of *flam* mutants

Genotype	Average no. of eggs laid/female/day	% sterility[a]	% fertility[b]
flam^{by+(P)}/*flam^{by+(P)}*	0	100	0
flam^{BG(P)}/*flam^{BG(P)}*	0	100	0
flam^{KG(P)}/*flam^{KG(P)}*	0.41	98.5	0.23
flam^{by+(P)}/*flam^{BG(P)}*	0.09	99.7	0.3
flam^{by+(P)}/*flam^{KG(P)}*	12	43	17.3
flam^{BG(P)}/*flam^{KG(P)}*	0.85	97.2	0
flam^{BG(P)}/*Def(1)l₃*	1.4	95	0.08
flam^{BG(P)}/*Def(1)l₁₅*	0.37	98	0.13
flam^{by+(P)}/*flam^{EY7(P)}*	15	40.3	14
flam^{BG(P)}/*flam^{EY7(P)}*	0	100	0
flam^{BG(P)}/*flam^{EY2(P)}*	0	100	0
flam^{BG(P)}/*flam^{EY18(P)}*	0	100	0

[a] $[1 - $ (no. of eggs laid/mutant female/no. of eggs laid/wild-type sibling)$] \times 100$. At least 100 3-day-old females were tested over a period of 9 days, at 22°.

[b] [no. of adult progeny/mutant female (9-day egg laying)/no. of adult progeny/wild-type sibling] $\times 100$.

between the two *flam* mutant phenotypes: sterility and loss of *gypsy* control.

We then tested two additional mutant strains for the permissivity and female sterility characteristics: *BG02658* contains the *P{GT1}* element (LUKACSOVICH *et al.* 2001) inserted 338 bp distally to the *P{lyB}* insertion site and *KG0476* carries the *P{SUPor-P}* element (ROSEMAN *et al.* 1995), 2.4 kb proximal to *P{lyB}* (Figure 1). The permissive/restrictive character was tested by monitoring the expression of a *gypsy-lacZ* transcriptional fusion in the FE (PELISSON *et al.* 1994). We found that both mutant lines are permissive and therefore we renamed them *flam^{BG(P)}* and *flam^{KG(P)}*. Most interestingly, both homozygous *flam^{BG(P)}* and *flam^{KG(P)}* females are sterile. This sterility is not fully complemented by *flam^{by+(P)}* (Table 1). *flam^{by+(P)}*/*flam^{BG(P)}* females produce very few eggs. There is partial complementation between *flam^{by+(P)}* and *flam^{KG(P)}* alleles. The number of eggs laid by *flam^{by+(P)}*/*flam^{KG(P)}* females is about half the number laid by wild-type sibling sisters. However, a high proportion of these eggs do not develop into adulthood. We could not test whether the permissivity of *flam^{BG(P)}* and *flam^{KG(P)}* females is caused by the insertions, as the *flam^{BG(P)}* parental strain (*Iso2A; Iso3A*; BELLEN *et al.* 2004) already carries a natural permissive allele and the *flam^{KG(P)}* parental strain is not available. From these experiments we conclude that the *flam* locus is required for female fertility. The three insertions present in *flam^{by+(P)}*, *flam^{BG(P)}*, and *flam^{KG(P)}* disrupt this oogenic function and, at least in the case of *flam^{by+(P)}*, also generate permissivity.

P-element-induced *flam* mutations reduce the accumulation of *gypsy* rasiRNAs in the ovary: We previously reported that antisense RNAs, 25–30 nucleotides in
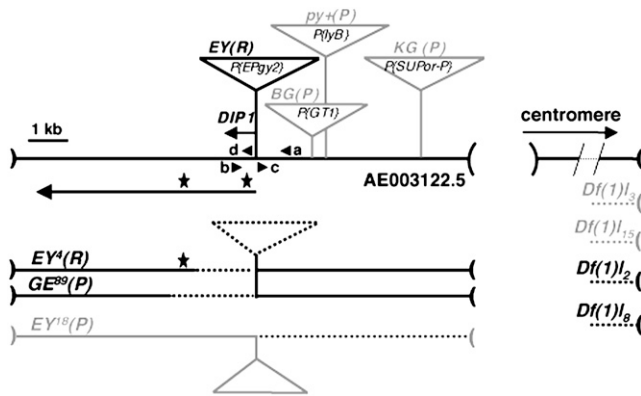
FIGURE 1.—Structure of the *DIP1-flamenco* region showing the positions of *P*-element-induced mutations and *X*-ray-induced deletions. The horizontal thick line represents sequenced DNA from scaffold AE003122.5, which extends between the *P{lyB}* insertion and a sequencing gap of unknown length (thin dotted lines). Positions of the *P*-element insertions within the region are indicated by a stalk and a triangle (not to scale). Insertions of *P{lyB}*, *P{GT1}*, and *P{SUPor-P}* generated, respectively, the *flam^{py+ (P)}*, *flam^{BG(P)}*, and *flam^{KG(P)}* female permissive and sterile alleles (shown shaded). Arrowheads a, b, c, and d indicate the positions and orientations of the primers used to analyze the excision events. The bent arrow indicates the *DIP1* transcription initiation site as deduced from the longest 5′ cDNA sequence (GenBank accession no. BI242976). Position and size of the *DIP1* transcription unit are indicated by a thick arrow. The two stars above the arrow indicate the position of the two DIP1 translation start sites. The *P{EPgy2}* insertion, 14 bp downstream of the *DIP1* transcription start site, generated the *flam^{EY(R)}* fertile allele. Below, the maps of deletions *EY4(R)*, *GE89(R)*, and *EY18(P)* are shown. Thick dotted lines indicate deletions. Distal deletions *EY4(R)* and *GE89(R)* are still fertile, while *EY18(P)* is female sterile (shown shaded). Also shown at the right are the maps of X-ray-induced deletions (ROBERT *et al.* 2001). The extremities of these deletions are not determined but their nearest breakpoint is located in the sequencing gap, >130 kb away from the *P[lyB]* insertion point.

length, complementary to *gypsy*, accumulate in ovaries of restrictive females, while the amount of such RNAs is dramatically reduced in females carrying a natural permissive allele (SAROT *et al.* 2004; PELISSON *et al.* 2007). These results strongly suggested that *gypsy* regulation is mediated by a rasiRNA pathway. We thus wondered whether in *P*-induced *flam* mutants the amount of *gypsy* rasiRNAs is affected. Comparison of the levels of *gypsy* rasiRNAs in *flam^{py+ (P)}/flam^{py+ (P)}* mutant and *flam^{FM7c(R)}/flam^{py+ (P)}* wild-type ovaries revealed that the *flam* mutation event resulted in a dramatic reduction of the amount of *gypsy* rasiRNAs (Figure 2). This effect does not result from the lack of development of *flam^{py+ (P)}* ovaries. Indeed, as we show below, homozygous *flam^{py+ (P)}* females, although they do not lay any egg, develop almost normal ovaries. The same significant reduction of *gypsy* rasiRNAs was also observed in a much less sterile genetic combination, *flam^{py+ (P)}/flam^{KG(P)}* (Figure 2 and Table 2). We conclude that *flam* is likely required for the accumulation of most, if not all, *gypsy* rasiRNAs in the ovary.
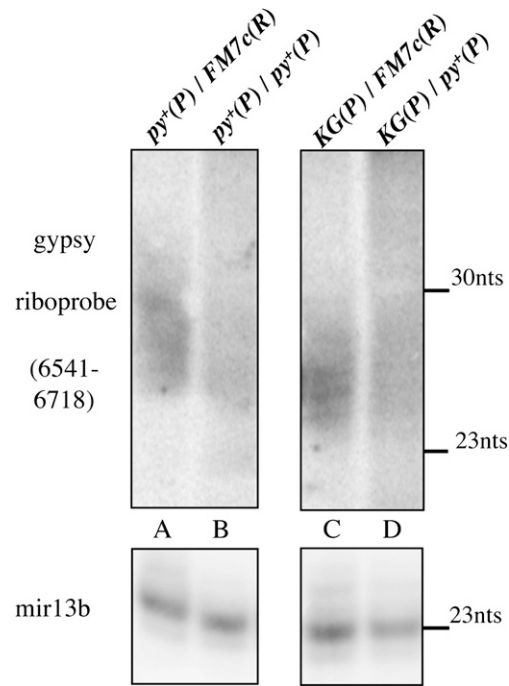

FIGURE 2.—*P*-element-induced *flam* mutations reduce the accumulation of *gypsy* rasiRNAs in the ovary. Low molecular weight-enriched RNA extracted from ovaries was hybridized (top) with a sense *gypsy* RNA probe (coordinates 6541–6718 in the *gypsy* sequence, accession no. M12027) and reprobed (bottom) with the end-labeled oligonucleotide complementary to the mir-13b miRNA. The position of the mir-13b 23-nt major band is indicated, as well as that of the 30-nt band of the Decade size marker (Ambion, Austin, TX). Antisense rasiRNAs, complementary to the *gypsy* probe were mostly detected in both genotypes containing the parental *FM7c(R)* restrictive chromosome, as compared with those containing its *py^+ (P)* mutant derivative.

The *flam* locus spans a large region of heterochromatin: To characterize the *flam* locus, we analyzed a series of deletions previously generated from a restrictive chromosome (PRUD'HOMME *et al.* 1995). The distal breakpoints of all these deletions are located >130 kb proximal to the *P{lyB}* insertion site (ROBERT *et al.* 2001; Figure 1). Two of them, *Df(1)l₃* and *Df(1)l₁₅*, have been shown previously to delete the ability of *flam* to repress *gypsy* expression and mobilization (ROBERT *et al.* 2001). Here we show that these deficiencies also delete the *flam* ovarian function since neither of them complements *flam^{BG(P)}* sterility (Table 1). In contrast, two other deficiencies, *Df(1)l₂* and *Df(1)l₈*, which remained restrictive, do complement *flam^{BG(P)}* sterility (Table 2). These results indicate that both functions of *flam* are disrupted by deficiencies located >130 kb proximal to the *P{lyB}* insert.

***DIP1* transgenes do not rescue *flam* functions:** The three inserts present in *flam^{py+ (P)}*, *flam^{BG(P)}*, and *flam^{KG(P)}* are located very close to the *DIP1* transcription start site (1.7 kb in *flam^{py+ (P)}*, 1.360 kb in *flam^{BG(P)}*, and 4.1 kb in *flam^{KG(P)}*; see Figure 1), suggesting that the *flam* mutant

TABLE 2

Mutations or reversions at the *flam* locus induce the simultaneous loss or gain of female fertility and ability to control *gypsy* and *ZAM*

flam allele	Complementation of *BG(P)* sterility	*gypsy* control	*ZAM* control
KG(P)	−	P	U
Iso2A; Iso3A[a]	+	P	S
BG(P)	−	P	U
FM7c(R)[b]	+	R	S
py+(P)	−	P	U
FM7c,679-5b(R)[b]	+	R	S
413(NP)[c]	+	R	S
l₂, l₈	+	R	S
l₃, l₁₅	−	P	U
PEY(R)[d]	+	R	S
EY(R)	+	R	S
EY4, EYn[e]	+	R	S
EY2, EY7, EY13, EY18	−	P	U
GE89(R)	+	R	S

P, permissive; R, restrictive; U, unstable; S, stable.

[a] An isogenized stock from which *BG(P)* originates (Bellen *et al.* 2004). It carries a spontaneous permissive allele.

[b] The *FM7c(R)* and *FM7c,679-5b(R)* X chromosomes, respectively, contain the parent and an excision revertant of the *py+(P)* mutant (Robert *et al.* 2001).

[c] The *413(NP)* X chromosome, restrictive for *gypsy* expression, has been used to generate the set of *lₓ* deficiencies (Prud'homme *et al.* 1995; Robert *et al.* 2001).

[d] The *X* parental chromosome of *EY(R)*.

[e] *EYn* stands for any of the 25 excision derivatives that remained fertile.

phenotypes displayed by these strains might result from *DIP1* inactivation. However, previous studies have shown that the *DIP1* transcription patterns of females carrying naturally permissive *vs.* restrictive *flam* alleles were not obviously different (Robert *et al.* 2001). This pattern is not affected in homozygous *flam^{py+ (P)}* mutant females either (data not shown).

We have previously shown that an 8-kb long genomic DNA transgene, potentially able to produce all *DIP1* transcripts, cannot rescue the *gypsy* regulatory function (Robert *et al.* 2001). The same was true for cosmid 7a, a larger genomic construct, extending 29 kb upstream of the *DIP1* transcription start site (Robert *et al.* 2001). Here, we performed appropriate crosses to yield homozygous *flam^{BG(P)}* or *flam^{py+ (P)}* females, carrying one or two copies of either transgene. Four independent transgenic lines carrying the 8-kb transgene and one line carrying cosmid 7a were tested. In none of these cases was the *flam* sterility phenotype rescued. We obtained the same negative results by heat-shocking at the L3-pupal stages two heat-shock constructs able to express one of the two longest DIP1 isoforms, DIP1-b and DIP1-c (see materials and methods).
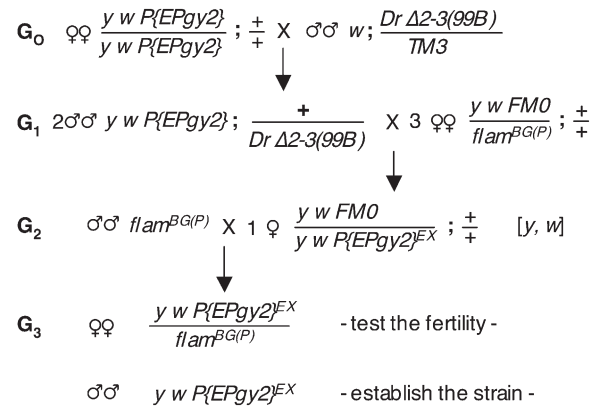

Figure 3.—Mating scheme used to select for excisions of the *P{EPgy2}* transposon from the *flam^{EY(R)}* strain. *FM0* and *TM3* are balancer chromosomes that are transmitted through meiosis with little or no recombination with the homologous chromosome. They carry the *B* and *Ser* markers, respectively, which, like *Dr*, are semidominant and can therefore be used to recognize and select for the appropriate chromosome combination in heterozygous flies. Mobilization of the *P{EPgy2}* transposon was induced in *flam^{EY(R)}* G1 males that had received the Δ2-3(99B) source of transposase. These males were then crossed to *FM0/flam^{BG(P)}* females and independent excision events were isolated in G2 as *FM0/flam^{EY}* females not expressing the *w* and/or *y* markers that indicate the presence of the *P{EPgy2}* insert. Viability of the excised alleles as well as their ability to complement *flam^{BG(P)}* sterility was tested in G3. All 130 tested alleles were viable.

DIP1 deletions do not affect *flam* functions: We analyzed a new strain, *EY02625* (renamed *flam^{EY(R)}*), containing the *P{EPgy2}* transposon inserted 14 bp inside the 5′ end of the longest *DIP1* cDNA (Bellen *et al.* 2004; Figure 1). We found that *flam^{EY(R)}* is not a *flam* mutant. It remained female fertile and restrictive like its parent *PEY* (Table 2). This result suggests that the region into which *P{EPgy2}* is inserted does not belong to the *flam* locus.

The possibility still exists, however, that the *P{EPgy2}* transposon does not inactivate *DIP1*. With the aim of inactivating this gene, we induced *P*-element imprecise excisions (see Figure 3). We obtained 130 independent excision derivatives of *flam^{EY(R)}* and analyzed them for their ability to complement the sterility of *flam^{BG(P)}*. For 30 of these derivatives we also amplified by PCR the genomic DNA flanking the *P{EPgy2}* transposon to molecularly characterize the excisions.

All derivatives were viable. For most of the 30 lines analyzed by PCR, a partial deletion inside the transposon was found, while flanking sequences were unaffected. Four excisants, *flam^{EY2(P)}*, *flam^{EY7(P)}*, *flam^{EY13(P)}*, and *flam^{EY18(P)}*, were homozygous female-sterile mutants that did not complement *flam^{BG(P)}* sterility (Table 1). Interestingly, these female-sterile excisants also simultaneously acquired the *flam* permissive character (Table 2). We analyzed by PCR 6 kb of sequence on each side of the initial insert. On the *DIP1* side, none of the four sterile

and permissive excisants exhibited any difference from the wild-type sequence. On the proximal side, except for *flam^{EY18(P)}*, we did not detect any alteration either. We assume that in these strains the mutational event that led to the *flam* mutant phenotype has taken place more proximally. The derivative *flam^{EY18(P)}* is different. In this case, the *P{EPgy2}* insert is still present, but proximally a large deletion, 133.9 kb long, has occurred (Figure 1). Indeed, the sequence of a 720-bp long genomic DNA fragment, flanking proximally the deletion, hits perfectly to Drosophila genomic sequences located 133.9 kb proximal to the *EY* insert (see supplemental data at http://www.genetics.org/supplemental/).

One derivative, *flam^{EY4(R)}* is particularly interesting. Proximally, the sequence is not affected. However, distally, 1.28 kb of the *DIP1* transcribed sequence has been deleted, removing the first putative translation start site (Figure 1). Nevertheless, *flam^{EY4(R)}* is fertile and restrictive like its parent. We also obtained a larger deletion, *flam^{GE89(R)}*, generated from the mutant *G1261* available from the Genexel company (see MATERIALS AND METHODS). As in *flam^{EY4(R)}*, the sequence is not affected proximally, but distally 1.9 kb of the *DIP1* transcribed sequence is deleted, removing the two putative *DIP1* translation start sites (Figure 1). Assuming that the following in-frame ATG could be used to initiate translation, *flam^{GE89(R)}* would produce truncated isoforms lacking the nuclear localization signal and a complete NH2 terminal domain. The DIP1-a, -b, and -c isoforms would be truncated by 69, 156, and 169 amino acids, respectively. Nevertheless, *flam^{GE89(R)}* is fertile and weakly restrictive like its parent (Table 2).

***flam* is necessary for ovarian germline stem cell differentiation and division:** Females of *flam* mutants exhibit atrophic ovaries, *flam^{BG(P)}* females showing the most severe phenotype. All ovaries of 24-hr-old *flam^{BG(P)}* females contain rudimentary ovarioles within which differentiation and division of germline cells is affected (Figure 4B). When differentiation takes place, ovaries become increasingly disorganized. Ovarioles contain less than three egg chambers and most egg chambers are characterized by a reduced number of degenerating nurse cells (Figure 4D). Cysts containing as few as one single nurse cell are frequently observed, suggesting that the ability of the differentiated cystoblasts to divide has been affected. The phenotypes presented by *flam^{by+(P)}* (Figure 5C) and *flam^{KG(P)}* (Figure 4C) mutant females are not as severe. Nevertheless, *flam^{KG(P)}*, like *flam^{BG(P)}* females, develop ovarioles that may be devoid of differentiating germline cells (Figure 4, B and C).

The very rare mature eggs that are formed in *flam^{BG(P)}* females present obvious defects in shape and dorsal appendage formation (Figure 4G). They are smaller than wild-type eggs and appear to lack dorsal–ventral and anterior–posterior polarities. Their dorsal appendages are shortened and abnormally shaped. Such defects could result from abnormal signaling from the FE.
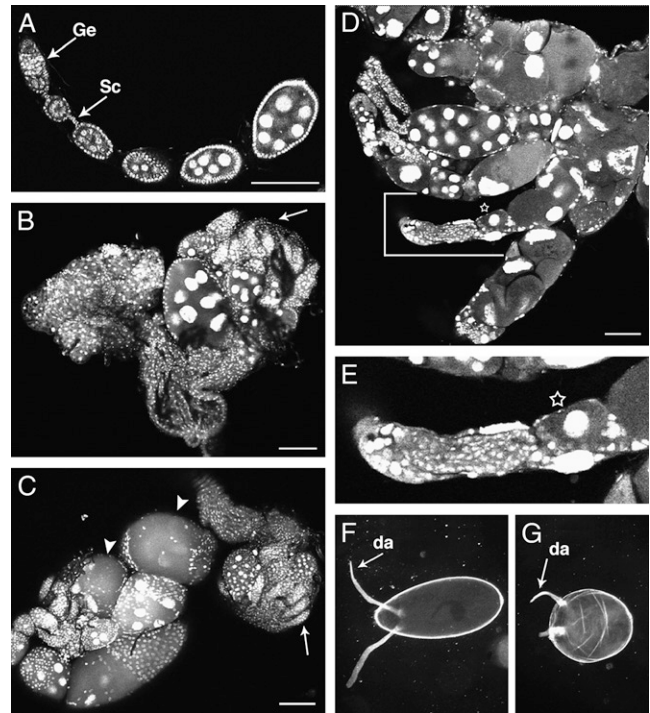

FIGURE 4.—*flam* mutations affect germline cell development. Ovaries were stained with DAPI. (A) A 24-hr-old wild-type ovariole contains a long string of developing egg chambers produced continuously by the germarium (Ge) and connected to each other by stalk cells (Sc). (B) *flam^{BG(P)}* and (C) *flam^{KG(P)}* are typical 24-hr-old ovaries. They are very small and contain a number of rudimentary ovarioles within which no differentiation of germ cells has taken place (arrows). In C two egg chambers exhibit gaps in the FE (arrowheads). (D) *flam^{BG(P)}* 48-hr-old ovarioles are severely dystrophic. Ovarioles contain two to three completely disorganized egg chambers. These chambers contain a reduced number of abnormally shaped nuclei. (E) Enlargement of the portion of ovariole shown in D, delimited by brackets. The star indicates an egg chamber containing a single nurse cell-like nucleus. (F) A wild-type mature egg. (G) A mutant *flam^{BG(P)}* mature egg lacks polarity and has abnormal dorsal appendages (da). Bars, 80 μm.

***flam* is somatically required for the formation of the follicular epithelium:** All three *flam^{by+(P)}*, *flam^{BG(P)}*, and *flam^{KG(P)}* alleles cause FE aberrations. In 6-day-old *flam^{BG(P)}* females (Figure 5A), the FE is completely disorganized. Large aggregates of cells can be observed between egg chambers while large gaps are formed above dying nurse cells. In some *flam^{KG(P)}* and *flam^{by+(P)}* mutant germaria, follicle cells probably fail to invaginate between the developing cysts. This results in composite egg chambers in which several germ cell syncytia are surrounded by a single follicle cell layer (Figure 5, B and C). Similar defects have been described for ovaries lacking the *fs(1)Yb*, *N*, *Dl*, *da*, or *brn* functions (RUOHOLA *et al.* 1991; GOODE *et al.* 1992; CUMMINGS and CRONMILLER 1994; JOHNSON *et al.* 1995). Defects in the FE of *flam^{by+(P)}* females are not as severe as those presented by *flam^{BG(P)}* or *flam^{KG(P)}* females. Sometimes the backward migration
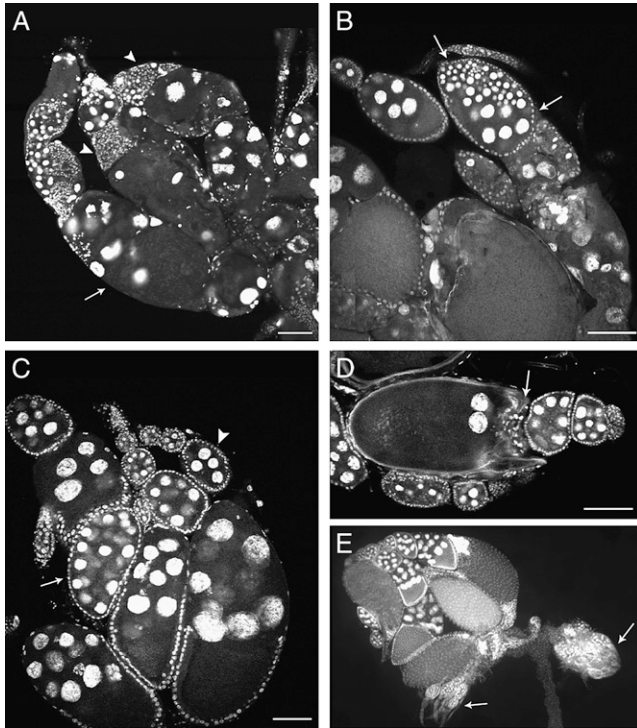
FIGURE 5.—Soma-dependent FE defects of *flam* mutants. (A) In 6-day-old *flam^BG(P)* ovaries, follicle cells form aggregates (arrowheads) while large gaps are seen elsewhere in the FE (arrow). (B) Forty-eight-hour-old *flam^KG(P)* ovarioles. Follicle cells fail to invaginate in the germarium, resulting in a compound egg chamber with nurse cells that present various degrees of polyploidization (arrows). (C) A 3-day-old *flam^py+ (P)* ovary. Abnormalities are frequently observed during ovarian development. Here a compound egg chamber is present (arrow). Also present is an egg chamber with a deficit in nurse cell number (arrowhead). (D) Stage 11 egg chamber from a 3-day-old *flam^py+ (P)* female in which follicle cells have not migrated properly, resulting in nurse cells being pinched off into the ooplasm. Follicle cells accumulate at the anterior of the egg (arrow). (E) A pair of ovaries containing ovarioles with homozygous *flam^BG(P)* germline clones and ovarioles from *ovo^D1 flam^+* germline cells. The *ovo^D1 flam^+* germline cells are arrested at the beginning of oogenesis because of the *ovo^D1* mutation (arrows). By contrast, in ovarioles in the left ovary, containing *flam^BG(P)* germline clones, germline stem cells divided normally. Bars, 80 μm.

of the columnar follicle cells is defective, with the result that a couple of nurse cells may be pinched off into the ooplasm (Figure 5D). Although a few mature egg chambers of normal appearance are found in *flam^py+ (P)* ovaries, they must be defective since no eggs are laid.

To determine if *flam* is needed in the germline or the soma, we produced mosaic females with germline cells homozygous for *flam^BG(P)* (the most severe *flam* mutation), while the somatic tissue carried one wild-type allele (PERRIMON and GANS 1983). We obtained fertile adult females that developed several completely normal ovarioles (Figure 5E). This result demonstrates that oogenesis occurs normally in *flam^BG(P)* germline clones. The effect of *flam* mutations on germ cell differentiation

described above must therefore reflect interactions between the germline and the defective ovarian somatic cells. Eggs produced from homozygous *flam^BG(P)* germ cell clones gave rise to normal adult flies. This result demonstrates that expression of *flam* in the germline, if any, does not provide an essential maternal contribution for embryogenesis.

***flam* also controls the expression of the *ZAM* retroelement:** The control of *ZAM*, another retroelement from *D. melanogaster*, appears to display strikingly similar features to that of *gypsy* (DESSET *et al.* 1999, 2003). *ZAM* expression is tightly repressed in most Drosophila strains (referred to as stable or S lines). However, in the Rev line, referred to as an unstable or U line, this control is disrupted, resulting in a high mobilization rate. The genetic determinant controlling *ZAM* mobilization is localized to the 20A region, the same region where *flam* maps (DESSET *et al.* 2003). Thus, we asked whether the *flam* mutants described above also disrupt *ZAM* control. We monitored *ZAM* expression with a *ZAM-LacZ* reporter containing the full-length LTR of *ZAM*. In the *U/U* background, *ZAM-lacZ* is expressed in the posterior follicle cells of all egg chambers, starting at stage 1, while in the *U/S* background, it is expressed only in stage 10 egg chambers (DESSET *et al.* 2003). As shown in Table 2 and Figure 6, loss or restoration of the female fertility function was always correlated with the corresponding loss or restoration not only of *gypsy* repression, but also of *ZAM* repression.

We also tested deficiencies *Df(1)l₃* and *Df(1)l₁₅* (Figure 1), which are permissive and sterile. We observed that *ZAM* control was lost in both deletions. By contrast, deficiencies *Df(1)l₂* and *Df(1)l₈*, which are restrictive and fertile, retained the ability to repress *ZAM* (Table 2). These results strongly suggest that Flam is necessary for three functions: ovary development, *gypsy* control, and *ZAM* control.

DISCUSSION

A strategy used by retroelements to gain access to the germinal cells is to be expressed specifically in the somatic tissue adjacent to the germline (LOCK *et al.* 1988; GODWIN *et al.* 1995). In *D. melanogaster*, *gypsy* clearly relies on its somatic expression in the FE to increase its copy number inside the germline (CHALVET *et al.* 1999). Indeed, not only are *gypsy* transcripts observed in FE cells, but also virus-like particles (LECHER *et al.* 1997). These are subsequently transferred to the oocyte before new copies of *gypsy* are inserted into the germline. This invasion is controlled by the *flam* locus. Here we show that *flam*, which represses the expression of *gypsy* in the FE, is required for morphogenesis of the FE and that it also controls the expression, in this tissue, of at least one other retroelement, *ZAM*.

***flam* fertility function is correlated with the ability to control *gypsy* and *ZAM*:** Both permissive and restrictive

parent
FM7c(R)

mutant
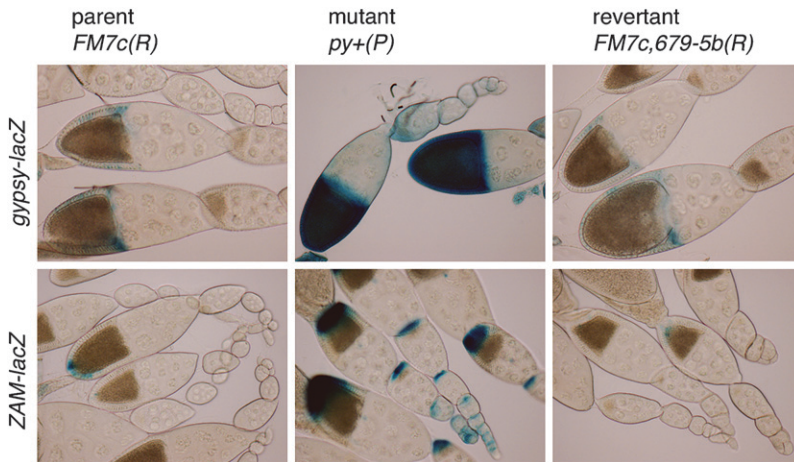py+(P)

revertant
FM7c,679-5b(R)

gypsy-lacZ

ZAM-lacZ

FIGURE 6.—*flam* controls the expression of both *gypsy* and *ZAM* retroelements. The parental *FM7c(R)* allele is restrictive, stable for *ZAM* expression (S), and female fertile (see Table 2). It represses the β-galactosidase expression driven by *gypsy* as well as *ZAM* LTRs. The *flam* female-sterile mutant allele, *py+(P)*, caused by insertion of the *P{lyB}* element into the *flam* locus of the *FM7c* chromosome, no longer represses the *gypsy* and *ZAM* reporters. In contrast, repression and fertility are restored in females carrying the revertant allele *FM7c,679-5b(R)*, generated by excision of the *P{lyB}* element.

flam alleles are present in natural populations (PELISSON *et al.* 1997), suggesting that they represent two functional allelic forms. We show in this article that permissive phenotypes can also be induced by mutations at the *flam* locus. Rearrangements at this locus, over a range of at least 130 kb, cause female sterility and simultaneously transform "restrictive-stable" alleles into "permissive-unstable" ones (*i.e.*, unable to repress both *gypsy* and *ZAM*). Although *flam* mutations cause defects in both the somatic and the germline components of the ovary, we demonstrate by germline clonal analysis that somatic expression of *flam* is sufficient to support oogenesis. This result indicates that the effect of *flam* mutations on female germ cell differentiation and division is most likely the consequence of aberrant somatic ovarian development. Thus, *flam* appears to be a large locus, at least 130 kb long, whose integrity is required for normal FE development and for control of the *gypsy* and *ZAM* retroelements.

DIP1 does not encode the Flamenco function: The two following observations led to the hypothesis that the *flam* function might be performed by *DIP1*. First, *P*-element insertions inactivating the *flam* restrictive function are all located upstream and close to the transcription initiation site of *DIP1*. Second, *flam*-dependent RNA silencing of *gypsy* (SAROT *et al.* 2004) might well be operated by the DIP1 protein that encodes two double-stranded RNA-binding domains (dsRBD) and binds dsRNA, a typical trigger for RNA silencing (DESOUSA *et al.* 2003).

However, our present results do not support this hypothesis. On the contrary, they suggest that *flam* is a large gene that extends proximal to *DIP1*. Indeed, the large *P{EPgy2}* transposon, present at the *DIP1* transcription start site, does not affect *flam* functions. The same is true for the deletions present in *flam^EY4(R)* and *flam^GE89(R)* that remove 1.28 and 1.9 kb, respectively, of *DIP1* transcribed sequences. Yet, in *flam^GE89(R)* the deletion removes the two translation start sites, presumably preventing translation of any of the known DIP1 iso-

forms. Conversely, all rearrangements occurring upstream of the *DIP1* transcription start site, located from a few kilobases up to >130 kb away, exhibit the *flam* mutant phenotypes. Identifying a transcription unit in this region has been, up to now, hindered by its repetitive nature and the presence of a sequencing gap of unknown size.

A model for the control of retroelements by *flamenco*: How could the *flam* heterochromatic gene perform such different functions such as the repression of retroelements and the control of FE development? Although both functions were always simultaneously affected by the rearrangements described in this article, they may be uncoupled from each other in natural allelic variants. Moreover, *gypsy* and *ZAM* retroelements can also be controlled independently from each other. Indeed, all the known natural *flam* alleles that are permissive for *gypsy* expression are stable, *i.e.*, able to repress *ZAM*. Symmetrically, the only spontaneous allele, known to derepress *ZAM*, restricts *gypsy* expression (DESSET *et al.* 2003). Furthermore, examples have been described of a permissive-stable strain becoming spontaneously restrictive stable (PRUD'HOMME *et al.* 1995) and of a restrictive-stable strain becoming restrictive unstable (DESSET *et al.* 2003). A model for *flamenco* should therefore explain both types of mutational events: on the one hand, the simultaneous loss of all functions, including fertility, resulting from genomic rearrangements at the *flam* locus, and, on the other hand, gain or loss of the ability to control either of the retroelements without loss of female fertility. The model should also take into account the fact that the *flam* locus of restrictive strains contains the genetic determinant(s) for the production of rasiRNAs that target any fragment of the *gypsy* genomic RNA (SAROT *et al.* 2004; PELISSON *et al.* 2007). At the molecular level, the recent *D. melanogaster* genome assembly (BDGP release 5) extends now 180 kb proximally to the *P{lyB}* insert. Most of this sequence consists of nested transposon remnants. Among them, it was of special interest to find two blocks of *gypsy* sequences, 2.4 and 3.2 kb long, that altogether might

accommodate most, if not all, of the *gypsy* rasiRNAs. In addition, this 180-kb fragment also contains a 5-kb block homologous to *ZAM*.

flam might be an RNA-silencing gene. Indeed, we know that a number of genes required for RNA interference (RNAi) are also involved in oocyte development (CHEKULAEVA and EPHRUSSI 2004). However, this hypothesis does not explain how three functions—FE development, regulation of the expression of *gypsy*, and *ZAM*—can be controlled by the same RNA silencing pathway independent of each other.

Alternatively, *flam* might correspond to heterochromatic proviral sequences, producing autonomously the RNA trigger(s) required for silencing of the homologous retrotransposon(s). This process could be similar to that reported for Mu killer, a naturally occurring transposon derivative in maize responsible for the silencing of the MuDr transposon family (SLOTKIN *et al.* 2005). Unlike Mu killer, the two putative *gypsy* triggers, found in the 180-kb sequence of release 5, do not have hairpin structures. The hypothesis of autonomously regulated RNA silencing triggers does not explain why the mutational insertions would abolish simultaneously the expression of all triggers. It does not explain either the sterility phenotype or why these sequences have to be localized at the *flam* locus rather than somewhere else in the genome.

Analyzing the genomic DNA sequence of the *flam* locus reveals striking features of this 180-kb heterochromatic chromosome region. Nearly all the retroelements present in the locus are oriented toward the telomere. We, thus, suggest a third hypothesis, assuming that the whole *flam* region is transcribed toward the centromere under the control of a single promoter. This promoter would cotranscribe a fertility gene and proviral sequences, among which are *gypsy* and *ZAM*, in an antisense orientation. This organization would be similar to that of the *Parp* gene, which spans >150 kb of centromeric heterochromatin and contains large introns accumulating various transposable elements, nearly all oriented opposite to the gene (TULIN *et al.* 2002). This hypothesis explains why *P*-induced female-sterile *flam* mutations also result in the failure of retroelement control: reducing the transcription level of this fertility gene would at the same time reduce the amount of RNA silencing trigger(s). This hypothesis also explains why such a variety of fertile natural *flam* alleles exist that are able to control or not able to control the expression of one or the other retrovirus, *gypsy* and *ZAM*. Such control would depend on the polymorphic presence, in putative *flam* introns, of *gypsy* and/or *ZAM* retroviral sequences. According to this hypothesis, the *flam* locus should be able to control the expression of other transposable elements.

We cannot exclude the possibility that this putative transcription unit does not contain any coding exon and that the sterility observed, in *flam*-mutant females, is the indirect consequence of high levels of transposition, resulting from the lack of rasiRNAs. This would be reminiscent of the indirect effect of mutants of the rasiRNA pathway on the specification of embryonic axis in Drosophila (KLATTENHOFF *et al.* (2007). Whatever the case, the localization of a transcribed locus in the pericentromeric heterochromatin could represent one of the ways used by organisms to defend themselves against retroviral invasion: the expression of such a locus in the very tissue used by retroviruses to gain access to the germline (here the FE) would be essential for triggering the repression of retroviral replication.

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