Evidence for a *piwi*-Dependent RNA Silencing of the *gypsy* Endogenous Retrovirus by the *Drosophila melanogaster flamenco* Gene

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ABSTRACT

In *Drosophila melanogaster*, the endogenous retrovirus *gypsy* is repressed by the functional alleles (restrictive) of an as-yet-uncloned heterochromatic gene called *flamenco*. Using *gypsy-lacZ* transcriptional fusions, we show here that this repression takes place not only in the follicle cells of restrictive ovaries, as was previously observed, but also in restrictive larval female gonads. Analyses of the role of *gypsy* cis-regulatory sequences in the control of *gypsy* expression are also presented. They rule out the hypothesis that *gypsy* would contain a single binding region for a putative Flamenco repressor. Indeed, the ovarian expression of a chimeric *yp3-lacZ* construct was shown to become sensitive to the Flamenco regulation when any of three different 5'-UTR *gypsy* sequences (ranging from 59 to 647 nucleotides) was incorporated into the heterologous *yp3-lacZ* transcript. The *piwi* mutation, which is known to affect RNA-mediated homology-dependent transgene silencing, was also shown to impede the repression of *gypsy* in restrictive female gonads. Finally, a RNA-silencing model is also supported by the finding in ovaries of short RNAs (25–27 nucleotides long) homologous to sequences from within the *gypsy* 5'-UTR.

RECENT genome sequence analyses have shown the quantitative importance of retroelements. For instance, genomic parasites, which replicate by retrotranscription and insertion of a cDNA elsewhere in the genome, make up >40% of the human genome (LANDER *et al.* 2001). The tight control of their mobilization is an important characteristic of their regulation to prevent high mutation rates due to novel insertions. Indeed, their transposition is usually a rare event. To study such regulations, fly geneticists have been paying special attention to some strains in which one or several elements display unusually high transposition rates. For instance, in the 2b3 (PASYUKOVA *et al.* 1997) and U (DESSET *et al.* 2003) unstable stocks, the *copia* and both the *Zam* and *Idefix* retroelements are mobilized, respectively. In two other strains, MG (MEVEL-NINIO *et al.* 1989; PRUD'HOMME *et al.* 1995) and MS (KIM *et al.* 1990), frequent transpositions of the *gypsy* element result in high levels of insertional mutability.

Gypsy was the first retrovirus described in invertebrates (KIM *et al.* 1994b; SONG *et al.* 1994). As shown in Figure 1A, the genomic structure of this retroelement is remarkably similar to the proviral form of vertebrate retroviruses (MARLOR *et al.* 1986). *Gypsy* proviruses contain two long terminal repeats (LTRs). Their transcription is initiated in the 5'-LTR by a TATA-less promoter, which contains a downstream promoter element (DPE) located 30 bp after the transcription start (ARKHIPOVA and ILYIN 1992). Transcription is terminated in the 3'-LTR,

giving rise to a full-length RNA. By analogy to vertebrate retroviruses, this RNA is thought to be used both as a messenger RNA, to translate, respectively, the Gag and Pol structural and enzymatic peptides, and as a genomic RNA, packaged into virus-like particles. The *env* messenger RNA that encodes the Env protein is a splicing derivative of the full-length *gypsy* transcript from which the *gag* and *pol* genes are excised (PÉLISSON *et al.* 1994). The expression of *gypsy* is restricted to the salivary gland precursors and fat body of the embryo, imaginal discs and fat body of larvae, and fat body and ovaries of adult females (SMITH and CORCES 1995). Both retrovirus-like organization and expression are typical of the functional *gypsy* proviruses that have inserted recently into the euchromatic DNA of some strains. By contrast, the pericentromeric heterochromatic regions of all strains contain very old *gypsy* insertions, which have accumulated a lot of deleterious mutations and rearrangements (LAMBERTSON *et al.* 1989; VAURY *et al.* 1989).

Genetic analysis of *gypsy* mobility has shown that it is controlled by an as-yet-uncloned locus of the *Drosophila* genome. This locus, *flamenco* (*flam*), is located in the heterochromatin of the X chromosome (PRUD'HOMME *et al.* 1995; ROBERT *et al.* 2001). Two classes of alleles, restrictive and permissive, have been described. Restrictive alleles, denoted *flam*^(R), are dominant and repress transposition of functional *gypsy* proviruses. Strains containing *flam*^(R) alleles are therefore stable, as far as *gypsy* mobilization is concerned. Conversely, permissive alleles, denoted *flam*^(P), allow high levels of *gypsy* transposition, as illustrated by the two unstable strains MG and MS, which are both *flam*^(P) homozygous and contain a

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large number of functional proviruses in their genome. Most of the permissive stocks are stable because they are devoid of functional *gypsy* proviruses (KIM *et al.* 1994a; PÉLISSON *et al.* 1997). A mutation from the restrictive toward the permissive allelic form was obtained by a *P*-element insertion (ROBERT *et al.* 2001). Moreover, deficiencies of the locus were shown to behave as permissive alleles (PRUD'HOMME *et al.* 1995). Hence, it is inferred that at least one of the functions of the putative Flamenco protein consists of downregulating *gypsy* mobilization. This is achieved by preventing the accumulation of first *gypsy* RNAs and then of virus-like particles into the somatic follicle cells of the restrictive ovaries (PÉLISSON *et al.* 1994; LECHER *et al.* 1997). However, it is not known whether the reduction of the steady-state level of *gypsy* RNAs in restrictive females occurs at the transcriptional or post-transcriptional level.

The purpose of the present work was to better understand the mechanisms of the *flam*-dependent regulation of *gypsy*. We started from a *gypsy-lacZ* construct containing the *gypsy* promoter and its 5'-untranslated region (UTR) fused to β -galactosidase. This transcriptional fusion can recapitulate the repression of the endogenous proviruses in the restrictive follicle cells (PÉLISSON *et al.* 1994). Assuming the hypothesis that *flam* would encode a typical repressor, this putative Flamenco protein would bind to a *cis*-regulatory sequence that we should be able to characterize by a deletion mapping study of this construct. Alternatively, by analogy to the *trans*-silencing phenomenon controlling the *P*element (RONSSERAY *et al.* 2003), the hypothetical Flamenco protein might be involved in some cosuppression-like mechanism that would target any construct containing homology to a putative *gypsy* trigger element. In this case, the *cis*-regulatory sequence analysis of the *gypsy-lacZ* construct should shed some light on the sequence content of this putative silencer *gypsy* element. Results from this analysis indicated that all the tested fragments originating from the *gypsy* 5'-UTR could be targeted by the Flamenco repression. The only apparent requisite was that the target had to be included in the tested transcript. This prompted us to further investigate a possible RNA-silencing model (i) by looking for the presence in restrictive ovaries of small interfering-like RNAs (DYKXHOORN *et al.* 2003), which, by virtue of their homology to the targets, should be able to guide the silencing complex toward the *gypsy*-containing transcripts, and (ii) by testing the impact on *gypsy* silencing of *piwi*, a gene known to affect RNA-mediated homology-dependent transgene silencing (PAL-BHADRA *et al.* 2002).

MATERIALS AND METHODS

Drosophila strains: If not otherwise indicated, genetic materials and fly stocks are described in FlyBase (<http://flybase.bio.indiana.edu/>). Flies were grown at 25° on standard *Drosophila* medium (GANS *et al.* 1975). The *piwi*² mutation, kindly pro-

vided by U. Bhadra, is a single *P-ry*¹¹ insertion (Cox *et al.* 1998). It was balanced with the *CyO; Act5c:GFP(w⁺)* chromosome constructed by D. Ferrandon. The permissive wOR(P) and the restrictive wRev(R) strains are both devoid of functional *gypsy* proviruses and were described previously (MEJLUMIAN *et al.* 2002). By contrast, all the following stocks contain active *gypsy* proviruses. The A151(P) X chromosome contains a permissive *flam* allele. It is maintained in the A151(P)/FM7(R) balanced stock where *gypsy* mobilization is prevented by the FM7 dominant restrictive balancer, as described previously (PÉLISSON *et al.* 1994). The A151(R) X chromosome was isolated as a restrictive derivative of the A151(P) chromosomal clone. The A151(R)/FM7(R) restrictive and the A151(P)/FM7(R) permissive balanced stocks were isogenized thereafter. The MG1 restrictive strain was described previously (PRUD'HOMME *et al.* 1995).

Plasmid constructions and *P*-element-mediated germline transformations: Several different *P*-element-containing plasmids were made, containing either the *gypsy* or the *yp3* promoter.

Constructs containing the *gypsy* promoter: The pGypLTR1 construct was kindly provided by V. G. Corces. This pUC18-based plasmid contains a *HpaI-XbaI* insert corresponding to nucleotides 5–435 in the *gypsy* sequence (accession no. M12927) and therefore exhibits the following simplified restriction map: *EcoRI-KpnI-SmaI/HpaI-BglII-XbaI*. It was used to extract the 5–430 *gypsy* fragment as a *EcoRI-KpnI-SmaI/HpaI-BglII* fragment, which was then inserted into the corresponding restriction sites of the *EcoRI-BglII-BamHI-PstI* transformation plasmid pW6 (KLEMENZ *et al.* 1987) to produce the p#6 construct. The *KpnI* site of p#6 was cut with *Acc65I* and filled with Klenow to obtain p#6ΔK. The *BamHI-KpnI-AUGlacZ-PstI* fragment of pCaSpeR-AUG- β gal (THUMMEL *et al.* 1988) was inserted into p#6ΔK at the corresponding restriction sites, *i.e.*, downstream of the *gypsy* LTR, to generate p#8. A 5'-UTR *gypsy* fragment (419–1072) was amplified from the plasmid containing the *gypsy* insertion into the *f'* mutant (MARLOR *et al.* 1986), with primers containing *BglII* and *KpnI* restriction sites (respectively, 5'-GAAGGAAGATCTCTAGACCTACT-3' and 5'-gggggtaccTGG TTGGCACACCACAAA-3', with non-*gypsy* sequences in lowercase and restriction sites underlined). After digestion with *BglII* and *KpnI*, the PCR product was ligated with the *BglII-KpnI*-digested p#8 plasmid to obtain p#12. The p#12Δ construct was obtained in two steps: The 59-bp deletion was first generated in the *gypsy* sequence of pGypLTR1 with the Stratagene (La Jolla, CA) QuikChange site-directed mutagenesis kit (primer: 5'-CGAAATAA ACCACAGCCCACAAGGCTAGTGATAATAACTAAGG-3'); the 435-bp *EcoRI-BglII* wild-type fragment of p#12 was then replaced by the corresponding 376-bp deleted fragment. The p#15 construct was obtained by replacing the *XhoI-KpnI* fragment of p#8 (which contains the 199–430 *gypsy* sequence) with the 199–328 *gypsy* sequence; the latter was provided by the *XhoI-KpnI*-digested PCR fragment that had been generated with the following primers on the p#12 template: 5'-ggaattCT CGAGGGTAAACTTAG-3' and 5'-gggggtaccatCGATAGCGAT TTGATTGT-3'.

Constructs containing the *yp3* promoter: The yp3fc plasmid was kindly provided by M. Bownes. This pBluescript-based plasmid contains in its *EcoRI* site the –285 to +43 fragment of the *yp3* gene, sufficient to drive expression in the follicle cells (RONALDSON and BOWNES 1995). The Stratagene QuikChange site-directed mutagenesis kit was used to introduce a *StuI* restriction site at coordinate –42 using the oligonucleotide 5'-GCAGTGCCTATCAGGCCTCGGAGCTATATAAG-3'. The pES2 parental construct was obtained by subcloning the 383-bp *BamHI-EcoRI-StuI-EcoRI-HindIII-XhoI-KpnI* mutated sequence upstream of *AUGlacZ* into the corresponding sites of the pCaSpeR-AUG- β gal transformation vector (THUMMEL *et al.*

1988). The 270–328 *gypsy* sequence was obtained by annealing the two corresponding oligonucleotides. The pES3 and pES4 plasmids were constructed by blunt-end cloning this 59-bp fragment at the *Stu*I restriction site of pES2 in the opposite and direct orientations, respectively. This small fragment was also ligated to the Klenow-blunted *Xho*I site of pES2 in the sense orientation to produce pES5. The Klenow-blunted *Xho*I site of pES2 was also used to insert the 329–426 and the 426–1072 *gypsy* fragments in the sense orientation, generating plasmids pES11 and pES9, respectively. Both these fragments originated from p#12 by either PCR or gel purification of a Klenow-blunted *Bgl*II-*Acc*65I fragment, respectively.

P-element-mediated transformations were performed as described (RUBIN and SPRADLING 1982). The constructs were coinjected with the *pUChs*IIΔ2-3 helper (gift of D. C. Rio) into embryos of the wOR(P) permissive stock. Flies carrying the insertion were identified by rescue of the *white* phenotype. Inserts in transgenic flies were made homozygous in this *w flam*^{OR(P)} permissive background. They were also backcrossed twice to wRev(R) females and then made homozygous in this restrictive background, too. Each pair of transgenes was analyzed (*Xba*I-restricted genomic DNA probed with the SV40 trailer) to check that the same transgene was indeed present in both backgrounds.

Histochemical analysis of β-galactosidase: Ovaries were fixed in 2% formaldehyde, 0.2% (vol/vol) glutaraldehyde/PBS for 5 min at room temperature, washed twice with PBS, rinsed once in staining buffer (1 mM MgCl₂/4 mM K₃Fe(CN)₆/4 mM K₄Fe(CN)₆/1% Triton X-100), and stained for 0.5–4 hr at 37° in staining buffer containing 0.27% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). For the sake of comparison, each pair of permissive and restrictive genotypes containing the same homozygous transgene was treated simultaneously. The reaction was stopped in PBS and the ovaries were mounted in 90% (vol/vol) glycerol in PBS and viewed in a Leica DMRB microscope.

Wandering female larvae were inverted after cutting off the head. They were treated as above except that the fixative concentration was reduced twofold, the X-gal concentration doubled, and the reaction stopped after 4 hr at 37°. After staining, females gonads were dissected out of the fat body in a drop of 90% glycerol and viewed directly in the dissecting microscope without mounting. Ovary and gonad photomicrographs were taken with a Coolpix990 digital camera (Nikon) and panels were constructed using Adobe Photoshop 5.5.

Sequence alignments: In an attempt to find common motifs that would explain why *gypsy* sequences 270–328, 329–426, and 426–1072 share the same regulatory potential, the sequences were compared using the following algorithms: Align (<http://www.infobiogen.fr>); Multalin (<http://probes.toulouse-inra.fr/multalin>); Alignment (<http://www.genebee.msu.su/cgi-bin/nph-malign.pl>); MAP Multiple Sequence Alignment (<http://searchlauncher.bcm.tmc.edu/cgi-bin/multi-align.pl>); EDTALN (<http://www.infobiogen.fr/services/analyseseq>); Clustalw (<http://www.infobiogen.fr/services/analyseseq>) and MEME (<http://meme.sdsc.edu/meme/website/meme.html>). Although the latter analysis did disclose a common motif of 10 nucleotides, this similarity turned out not to be significant when compared to the results of the shuffled run.

Detection of small RNA species: The detection of small RNA was performed essentially according to HAMILTON and BAULCOMBE (1999) with the following modifications: RNA isolated from 25 females using the Trizol reagent was enriched in low-molecular-weight species by precipitation in 5% polyethylene glycol 8000/0.5 M NaCl, loaded in each lane of a 15% denaturing acrylamide:bisacrylamide (19:1) gel, transferred onto Hybond NX membrane (Amersham, Buckinghamshire, UK), and fixed by ultraviolet crosslinking. Before transfer, the

gel was imaged after staining in 2 μg/ml ethidium bromide. Hybridization with a short sense *gypsy* riboprobe (coordinates 310–433 in the *gypsy* sequence) was performed overnight at 40°; the membrane was washed twice for 30 min at 50° in 2× SSPE/0.5% SDS and exposed for 2 days on a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA). The blots were stripped for 1 min at 90° in 0.2% SDS/10 mM Tris pH 7.5 and probed with an end-labeled oligonucleotide (5'-ACTCGT CAAAATGGCTGTGATA-3') complementary to the mir-13b-1 microRNA.

RESULTS

Larval female gonads also display a *flam*-dependent *gypsy* expression: The p#12 construct contains the promoter and the 5'-UTR of *gypsy*, transcriptionally fused to the *Escherichia coli lacZ* reporter (Figure 1B). It is very similar to the previously described pgypCaSpeR plasmid (SMITH and CORCES 1995), which is known to have a *flam*-dependent expression (PÉLISSON *et al.* 1994). Three transgenic insertions, #12a, -b, and -c, were obtained from this construct and were checked for display of the expected *flam*-dependent expression in the ovary: *lacZ* staining was strong in follicle cells of the permissive, but not the restrictive, egg chambers at stages 8–10 (Figure 1, B and C, left). Moreover, the female gonads of third instar larvae were also found to respond to the Flamenco regulation (Figure 1D): *lacZ* staining was observed only in the permissive, not the restrictive, gonads.

The repression of *gypsy* by the putative Flamenco protein does not require the *Su(Hw)*-binding region: Upon binding to the insulator located in the 3' half of the *gypsy* 5'-UTR, the *Su(Hw)* protein has been shown to activate the ovarian β-galactosidase expression of the pgypCaSpeR *gypsy-lacZ* transgenes (SMITH and CORCES 1995). This experiment was done in the *Df(1)w, y w^{67c23}* strain, which has since been shown to be a permissive *flam* mutant (data not shown). We therefore wondered whether the enhancer activity of the *Su(Hw)* protein would also have been observed in a restrictive genotype. In other words, could the repressive function of *flam* be mediated by some competition with the enhancer located in the *Su(Hw)*-binding region? This hypothesis was clearly ruled out by the observation that the p#15 deleted construct is sensitive to the Flamenco repression in ovaries, even though it is missing the *Su(Hw)*-binding region (Figure 1, B and C, right). As expected, in the permissive background, the #15 transgenes were consistently less expressed than the #12 transgenes. However, since the p#15 deletion also uncovers upstream sequences, this effect might be due to more than the mere absence of the *Su(Hw)*-binding region. The expression of the #15 transgenes in permissive female gonads was too low for their regulation to be tested in the restrictive background (data not shown).

The restrictive repression does not require the *gypsy* promoter: The p#15 deleted construct is a good target

FIGURE 1.—Transgenes containing the first 328 nucleotides of *gypsy* are sensitive to the *flam*-dependent regulation. (A) Structure of the *gypsy* proviral DNA and schematic of the *gypsy* promoter. Coding regions of three genes, *gag*, *pol*, and *env*, are shown by open boxes. Shaded boxes represent the 482-bp long terminal repeats (LTR). *Gypsy* has a TATA-less promoter, which contains a DPE located 30 bp after the transcription start (ARKHIPOVA and ILYIN 1992). The broken arrow indicates the starting point and the orientation of transcription. The Su(Hw)-binding region is shaded in yellow and is denoted “Su(Hw) B.R.” (B) Structure and regulation of the p#12 and p#15 constructs. p#12 is a transcriptional fusion of the *gypsy* promoter and leader with the prokaryotic *lacZ* reporter gene. The p#15 deletion derivative includes nucleotides 329–1072. In the inset table, the total numbers of transgenes that were made homozygous in both the *w flam^{OR(P)}* permissive and the *w flam^{Rev(R)}* restrictive backgrounds are given in

parentheses in addition to the number of regulated transgenes (*i.e.*, those disclosing a markedly lower expression in the restrictive than in the permissive genotype). (C) *flam*-dependent follicle cell expression of two homozygous transgenes, #12a and #15(2), representative of the data obtained with the p#12 and p#15 constructs. The blue β -gal staining is restricted to the permissive follicular epithelium that surrounds the grayish yolk-containing oocyte. (D) *flam*-dependent female gonadal expression of the #12a homozygous transgene. Third instar female larvae contain acorn-like small gonads. In the permissive gonad, *lacZ* is expressed nonuniformly throughout the “acorn-cup,” including the equatorial region known to contain both precursors of the germinal and somatic cells of the ovary. The histochemical reaction was stopped after 4 hr at 37°.

for the restrictive function despite the fact that it contains only 328 bp of *gypsy* sequence, corresponding mostly to the promoter (coordinates 1–270) followed by ~60 bp of nonpromoter transcribed sequences (Figure 1, A and B). To test whether the *gypsy* promoter is dispensable for this regulation, we chose to swap it for an alternative promoter from the *yp3* gene. The enhancer located immediately upstream of this promoter is known to drive expression in the follicular epithelium of stage 10 egg chambers (RONALDSON and BOWNES 1995), where *gypsy* itself is expressed. As a first control, we checked that the pES2 corresponding reporter construct was not repressed in a restrictive background (Figure 2, B and C). Then the nonpromoter *gypsy* sequence present in p#15 (270–328) was introduced downstream of this *yp3* ovarian promoter in the sense orientation. The resulting pES5 construct was repressed by the re-

strictive *flam* genotype (Figure 2, B and C), indicating that this small 59-bp fragment is sufficient to make a non-*gypsy*-driven transcription unit sensitive to this regulation.

When fused to a heterologous transcript, any fragment from the *gypsy* 5'-UTR appears to be able to target the repression: To determine whether this 59-bp *cis*-regulatory sequence is the only possible target of the *flam*-dependent regulation, we specifically deleted it from the original p#12 *gypsy* construct. As shown in Figure 2, B and C, this deletion did not prevent the #12 Δ transgene from being repressed in the restrictive background, suggesting some level of redundancy in the *gypsy* control mechanism.

As a first step to dissect the target sequence(s) left in the p#12 Δ construct, the nonpromoter sequence present in this construct (329–1072) was split into two frag-

FIGURE 2.—Any fragment from the *gypsy* 5'-UTR, when inserted into the *yp3-lacZ* heterologous transcription unit, can be a target of the *flam*-dependent regulation. (A) Coordinates in the *gypsy* sequence of each of the three fragments tested. These three fragments scan the whole nonpromoter *gypsy* sequence present in the p#12 construct. (B) Structure and regulation of the constructs. p#12Δ differs from p#12 only by the deletion of nucleotides 270–328. pES2 is a transcriptional fusion of the *yp3* minimal ovarian promoter (cross-hatched fragment containing the ovarian enhancer shown by an open box) with the prokaryotic *lacZ* reporter gene. pES2 contains two unique restriction sites (*Stu*I and *Xho*I) that were used to insert one of the three different *gypsy* fragments upstream or downstream of the promoter, giving rise to the five other pES constructs. In the inset table the numbers of the corresponding transgenes that were sensitive to the Flamenco repression are given (as in Figure 1). (C) Representative examples of the expression of four of the seven constructs in adult ovaries. For each construct, a pair of stocks homozygous for a given transgene in both the permissive and restrictive genotypes is presented.

ments (329–426 and 426–1072), which were both tested in the same way as the 59-bp fragment. Both resulting transgenes (ES11 and ES9) displayed typical regulation

(Figure 2B), indicating the existence of at least three different target sequences in the 5'-UTR of *gypsy*. Several unsuccessful attempts were made to align these three

sequences (see MATERIALS AND METHODS), making very unlikely the hypothesis that they would share redundant binding sites for a repressing transcription factor putatively encoded by *flam*. The only feature common to the *gypsy* sequences present in pES5, pES9, and pES11 might be that each of them would be homologous to some hypothetical *gypsy* element(s) that would trigger a homology-dependent silencing-like mechanism.

This putative trigger would target RNA rather than DNA, since the 59-bp sequence does not behave as a target when inserted upstream of the same *yp3* promoter (see the ES3 and ES4 transgenes in Figure 2, B and C).

The repression of *gypsy* is impaired in *piwi* mutant larval female gonads: The *Drosophila argonaute*-like *piwi* gene was reported to be involved in the *Adh* transgene cosuppression (PAL-BHADRA *et al.* 2002). Hence, we addressed the question of whether *piwi* would also affect the apparently similar phenomenon of *gypsy* silencing. Following the mating scheme described in Figure 3, the *piwi*² mutant allele was introduced into a restrictive background homozygous for the #12a *gypsy-lacZ* transgene. The stock thus obtained generates both homozygous and heterozygous *piwi* females, which can be distinguished, at any developmental stage, by the green fluorescent protein (GFP) fluorescence carried by the latter on their *CyGFP* balancer chromosome. The restrictive genotype of this stock was confirmed by the absence of any *lacZ* expression in the heterozygous adult ovaries (data not shown). The morphology of homozygous *piwi*² ovaries is too severely affected for any stage 10 to be recognized and the *lacZ* expression to be monitored in the corresponding follicle cells. We took advantage of the larval gonadal *gypsy* regulation described above to study the impact of the *piwi*² mutation. As expected, no staining was observed in the heterozygous third instar larval female gonads, confirming the restrictive genotype of the stock (Figure 3). By contrast, homozygous *piwi*² gonads disclosed *lacZ* expression in spite of their restrictive genotype. The staining pattern appeared to correspond to the apical-most part of the pattern observed for *gypsy* expression in a *flam* permissive genotype (see Figure 1D). This derepression suggests that the mechanism of *gypsy* silencing involves the Piwi function.

Evidence for small *gypsy* RNAs: Collectively, these data (involvement of Piwi and redundancy of the *gypsy* targets and their apparent need to be transcribed) provide circumstantial evidence for a homology-dependent RNA-silencing mechanism triggered by unknown *gypsy* sequence(s). Such a hypothesis assumes the existence of antisense small *gypsy* RNAs, which should mediate the silencing information between the hypothetical trigger and the targets. This prediction was tested by Northern blot hybridization with a riboprobe from within the *gypsy* 5'-UTR (coordinates 310–433). Small RNAs (25–27 nt) were easily detected in the restrictive ovaries and whole females (Figure 4). The signal disclosed by both types

FIGURE 3.—*Gypsy* derepression in restrictive *piwi* mutant larval female gonads. (A) Mating scheme to produce a *wRev(R)* restrictive balanced stock containing both a *piwi*² mutation and the #12a regulated transgene. Three markers were used to keep track of the various chromosomes: GFP stands for the *Act5c:GFP(w⁺)* construct, which can be viewed by epifluorescence at any postembryonic developmental stage; the brown-eyed phenotype of this transgene is not epistatic over the red-eyed color of the #12a transgene; and the *TM3* balancer was followed by the *Sb* marker. (B) Derepression of the #12a transgene in *wRev(R)* restrictive gonads homozygous for the *piwi*² mutation. The histochemical reaction was stopped after 4 hr at 37°. The *lacZ* staining is less widespread than that in the corresponding permissive *piwi*⁺ gonads (see Figure 1D). It is mostly located in the central region of the gonad. Gonads were photographed and imaged exactly like those in Figure 1.

of permissive females tested here, A151(P) and *wOR*(P), was about twofold fainter than that of their isogenic restrictive counterparts, A151(R) and *wRev*(R), respectively. However, in view of the large interstrain variations, it would be premature to infer that the *flam* genotype affects the abundance of the small *gypsy* RNAs.

FIGURE 4.—Detection of small RNAs homologous to sequences from within the *gypsy* 5'-UTR. Low-molecular-weight-enriched RNAs extracted from either ovaries (A) or whole females (B) were hybridized with a sense *gypsy* riboprobe (coordinates 310–433 in the *gypsy* sequence; accession no. M12927). The names of the strains studied in this experiment are indicated above the corresponding lanes; A151(R)/A151(R) and A151(P)/A151(P) homozygous females were isolated from the A151(R)/FM7(R) and A151(P)/FM7(R) balanced stocks, respectively (see MATERIALS AND METHODS for a description of these strains). After stripping, the membranes were probed with an end-labeled oligonucleotide complementary to the abundant mir-13b-1 microRNA. The sizes of most *gypsy* small RNAs were estimated to range between 25 and 27 by extrapolation of this ladder of bands step by step down to the position of this 22-nt microRNA. Thirty nucleotides corresponds to the approximate position of the 2S rRNA as visualized by ethidium bromide staining before transfer.

DISCUSSION

Tissue specificity of the *flam*-dependent control of *gypsy*: In adult permissive females, *gypsy-lacZ* transgenes are expressed in both the fat body and the ovarian follicle cells, but the repression is observed only in restrictive ovaries, not in restrictive carcasses (PÉLISSON *et al.* 1994). We report here another tissue-specific restriction of the *flam*-dependent control of *gypsy*, namely of the third instar larval female gonad. Other larval tissues, such as the imaginal discs and larval fat body (SMITH and CORCES 1995), do exhibit some expression of the *gypsy-lacZ* transgenes but, unlike the female gonad, they

are not sensitive to the *flam* genotype (data not shown). *Gypsy* is expressed in only the basal half of the gonad. This *flam*-dependent expression pattern includes the central region, which is known to contain both precursors of the germinal and somatic cells of the ovary (GODT and LASKI 1995). Since we have not yet identified this new *gypsy*-expressing cell type, it remains to be seen whether or not this *flam*-dependent control of *gypsy* is also restricted to the somatic cells, as it is in the adult ovary.

Circumstantial evidence for a cosuppression-like mechanism involved in the *flam*-dependent control of *gypsy*: The experiments presented in this article were initiated to test the hypothesis that the control of *gypsy* would operate via a classical transcriptional repressor encoded by *flam*. This simple hypothesis was systematically ruled out by each of the five following lines of circumstantial evidence:

- i. The *gypsy* promoter sequences are not required for this regulation to operate. The *yp3* promoter belongs to a different class from that of the *gypsy* promoter (presence of a TATA box and absence of a DPE) and does not display a *flam*-dependent expression. Nevertheless, the *yp3-lacZ* heterologous transcription unit could be made sensitive to the Flamenco repression by incorporation of nonpromoter *gypsy* sequences.
- ii. Three nonhomologous fragments of the *gypsy* 5'-UTR were shown to be good targets of the repression. Since these fragments do not share any common sequence, the three corresponding hypothetical Flamenco binding sites, scattered over several hundreds of nucleotides, should be very degenerate, although functionally redundant (each sequence is indeed able, by itself, to mediate a strong level of repression).
- iii. Unlike the usual silencers, these regulatory sequences apparently need to be located downstream of the heterologous promoter, suggesting that they have to be transcribed and targeted as RNA.
- iv. Small RNAs (25–27 nt) were detected in ovaries by hybridization to a 120-nt probe from the *gypsy* 5'-UTR. This class of RNAs is considered a hallmark of RNA-mediated silencing phenomena in eukaryotic organisms (HANNON 2002). It is worth noting that RNA silencing is considered an adaptive defense mechanism developed by eukaryotic genomes to control genetic parasites like viruses and transposable elements (WATERHOUSE *et al.* 2001).
- v. Finally, the involvement in this regulation of the argonaute-like *piwi* gene, which was already reported to affect *Adh* cosuppression (PAL-BHADRA *et al.* 2002), is additional circumstantial evidence for a RNA-mediated cosuppression mechanism. Although of unknown biochemical function, several

members of the Argonaute gene family are indeed among the most conserved universal components of the RNA-silencing process (CARMELL *et al.* 2002). If this effect of the pleiotropic *piwi* mutation is not indirect, it might provide the first hint for a role of an Argonaute-like gene in the control of transposable elements.

About the putative trigger(s) of this hypothetical co-suppression phenomenon: In the aforementioned *Drosophila* cosuppression model, multicopy *Adh* transgenes can trigger the repression of the endogenous *Adh* gene together with their own repression. In the case of the *gypsy* regulation, we do not know what the trigger(s) would look like, except that it (they) should at least contain some homology to each of the three fragments identified as regulatory targets in the *gypsy* 5'-UTR. Since the repression can be observed even in those restrictive strains which, like wRev(R), are devoid of active *gypsy* proviruses, trigger(s) must be searched for among the defective elements shared by all strains and located mainly in the pericentromeric heterochromatin (LAMBERTSSON *et al.* 1989; VAURY *et al.* 1989). In *Drosophila*, cosuppression is involved in the repression of several transposable elements and repeated sequences, including the *P* element (RONSSERAY *et al.* 2003), the *Stellate* repeats (ARAVIN *et al.* 2001), and the *I* element (CHABOISSIER *et al.* 1998; JENSEN *et al.* 1999; MALINSKY *et al.* 2000; ROBIN *et al.* 2003). The fact that heterochromatic *gypsy* elements might be responsible for the silencing of the euchromatic ones recalls that, in the case of *P* and *Stellate*, the triggers are located in the telomeric and the pericentromeric heterochromatin, respectively. In the case of the *I* element, it is worth noting that pericentromeric ancestral *H*-like sequences were also suggested to relay *I*-element cosuppression (BUCHETON *et al.* 2002; JENSEN *et al.* 2002).

Transcriptional vs. post-transcriptional silencing: Transposable elements are known to be repressed at both transcriptional (OKAMOTO and HIROCHIKA 2001) and post-transcriptional levels (WU-SCHARF *et al.* 2000). The *piwi* *Drosophila* gene is required not only for the post-transcriptional but also for some aspects of the transcriptional *Adh* cosuppression phenomenon (PAL-BHADRA *et al.* 2002). Moreover, recent data indicate that RNA silencing may also result in transcriptional inactivation mediated at the level of chromatin (VOLPE *et al.* 2002). Thus, the effect of the *piwi*² mutation cannot unambiguously relate to the level at which the *gypsy* silencing acts and neither can the apparent need for transcription of the *gypsy* target, nor even the presence of short *gypsy* RNAs. The differential sensitivity of the spliced and unspliced *gypsy* RNAs to the restrictive repression (PÉLISSON *et al.* 1994) would support the post-transcriptional hypothesis, but this question awaits direct testing by experiments such as run-off transcription of ovarian nuclei.

Roles of *flam* and *piwi* in this silencing: The present results suggest that RNA silencing might be responsible for the repression of *gypsy* in the ovaries that contain functional *flam* and *piwi* alleles. Assuming this hypothesis, it is not clear whether *flam* is involved in the accumulation of the short *gypsy* RNAs. It is difficult to define a role for *flam* in this process until it has been clearly identified at the molecular level. It is nevertheless striking that inactivation of this restrictive function was caused by a *P*-element insertion (ROBERT *et al.* 2001) just 1.7 kb upstream of a gene, *DIP1*, which was subsequently shown to bind double-stranded RNA (DESOUZA *et al.* 2003), a typical trigger of RNA silencing. *DIP1* discloses a ubiquitous expression pattern, including the ovarian tissues in which the Flamenco repression was shown to operate.

PIWI belongs to a highly conserved protein family that is involved in a variety of RNA-silencing phenomena in a diverse set of organisms (CARMELL *et al.* 2002). PIWI itself affects the *Adh* transgene cosuppression process (PAL-BHADRA *et al.* 2002). To know whether both genes are involved in the same regulatory pathway(s), we are planning to monitor the level of *gypsy* expression in the *flam piwi* double mutant. It would also be interesting to test whether *flam* affects *Adh* cosuppression, as well as other RNA silencing phenomena. Finally, when the *flam* product(s) can be detected, it will be worth looking at the possible effect of *piwi* on the expression of *flam* itself.

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