A Novel Repeat-Associated Small Interfering RNA-Mediated Silencing Pathway Downregulates Complementary Sense *gypsy* Transcripts in Somatic Cells of the *Drosophila* Ovary[▽]

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Replication of the *gypsy* endogenous retrovirus involves contamination of the female germ line by adjacent somatic tissues. This is prevented by *flam*, an as-yet-uncloned heterochromatic pericentromeric locus, at the level of transcript accumulation in these somatic ovarian tissues. We tested the effect of a presumptive RNA silencing mechanism on the accumulation of RNAs produced by constructs containing various *gypsy* sequences and report that the efficiency of silencing is indeed correlated with the amount of complementary RNAs, 25 to 30 nucleotides in length, in the ovary. For instance, while these RNAs were found to display a three- to fivefold excess of the antisense strands, only the transcripts that contain the complementary sense *gypsy* sequences could be repressed, indicating that they are targeted at the RNA, not DNA, level. Their size and asymmetry in strand polarity are typical of the novel repeat-associated small interfering RNA (rasiRNA)-mediated pathway, recently suspected to prevent the deleterious expression of selfish DNA specifically in the germ line. Unlike microRNAs (but like rasiRNAs and, surprisingly, siRNAs as well), *gypsy* rasiRNAs are modified at the 3' end. The rasiRNA-associated protein Piwi (but not Aub) is required for *gypsy* silencing, whereas Dicer-2 (which makes siRNAs) is not. In contrast, *piwi*, *aub*, and *flam* do not appear to affect somatic siRNA-mediated silencing. The amount of *gypsy* rasiRNAs is genetically determined by the *flam* locus in a provirus copy number-independent manner and is triggered in the somatic tissues by some pericentromeric provirus(es), which are thereby able to protect the germ line from retroviral invasion.

RNA interference (RNAi) is a common reverse-genetics method that can be used to silence genes in most eukaryotes and involves the introduction of double-stranded RNA molecules (dsRNA) that are complementary to the targeted genic sequences (16). This technique takes advantage of well-conserved, natural RNA silencing mechanisms in which RNase III endonucleases (8) cleave dsRNAs into 21-nucleotide (nt) small RNAs, called small interfering RNAs (siRNAs). siRNAs interact with proteins of the Argonaute family to confer sequence specificity to the silencing complex (55).

Various naturally occurring siRNA-like molecules have been described in eukaryotes. These include (i) animal microRNAs (miRNAs), the sequences of which can be conserved from worms to humans (29, 40); (ii) scanRNAs in *Tetrahymena thermophila* (36) and centromeric repeat-originating siRNAs in *Schizosaccharomyces pombe* (45); and (iii) siRNAs produced by viruses in plants (20), transposons in *Caenorhabditis elegans* (56) and transposons in protozoa (14, 61). The silencing abilities of these natural small RNAs are involved in diverse biological processes, such as (i) the regulation of gene expression by translational inhibition (2), (ii) genome organization and chromosome structure mediated by chromatin modification (60, 65), and (iii) defense against viruses and invasive repeated

DNAs (64). Several possible triggers for the production of their dsRNA precursors have been identified, including the folding back of inverted repeats (made possible, for instance, by the palindromic organization of the primary miRNAs), the activity of RNA-dependent RNA polymerases (11, 13, 34, 38, 57, 59), and readthrough antisense transcription of repeated DNA sequences (5, 56).

In *Drosophila melanogaster*, there is increasing evidence that some proteins thought to be involved in RNA-silencing mechanisms are required for the repression of retrotransposable elements (7, 22, 26, 52, 53, 62). At least one-fourth of all natural small RNAs cloned in *Drosophila* (6) were homologous to one or another family of repetitive DNA (mostly retroelements). These so-called repeat-associated siRNAs (rasiRNAs) form a new class of small RNAs, characterized in particular by their slightly longer size. However, direct evidence for their involvement in homology-dependent silencing of retroelements is still lacking. The data presented here indicate that the *gypsy* endogenous retrovirus of *D. melanogaster* is repressed in ovarian somatic cells by a rasiRNA-mediated RNA silencing pathway that is different from those of miRNAs and siRNAs.

Functional *gypsy* proviruses are very similar, in structure and expression, to simple vertebrate proviruses. They contain a single promoter that drives the expression of the Gag, Pol, and Env polypeptides (Fig. 1A and B). The endogenous replication of *gypsy* is repressed by the as-yet-uncloned heterochromatic *flamenco* (*flam*) gene (46). This host resistance gene is highly polymorphic (42) and controls *gypsy* expression in the somatic follicle cells of the ovaries. A single restrictive *flam* allele is sufficient to reduce the accumulation of *gypsy* transcripts in the follicle cells (41). In contrast, functional *gypsy* proviruses can

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FIG. 1. Schematic representation of the probes, constructs, and functional *gypsy* proviruses and transcripts. (A and B) Conserved structure of active *gypsy* proviruses and their genomic and subgenomic transcripts. The three ORFs (*Gag*, *Pol*, and *Env*) are indicated by light gray boxes, as are both 482-bp long terminal repeats (LTRs). The black and hatched boxes represent the small fragments inserted into the pES5 and pESpol constructs schematized below. The scheme is drawn to scale. (C) Sense and antisense RNA probes. The coordinates of the PCR fragments used to synthesize labeled RNA are from the *gypsy* sequence (GenBank accession number M12927). (D) Structure of the constructs. pES2 is a transcriptional fusion of the minimal ovarian *yp3* promoter (dark gray box) with the prokaryotic *lacZ* reporter gene. It contains a unique *XhoI* restriction site in the 5'UTR that was used to insert small *gypsy* fragments in the indicated orientations, giving rise to the pES5, pESpol+, and pESpol- constructs.

replicate in the females of permissive strains because the permissive *flam* alleles present in these strains lack the repressive function of *flamenco* (44). The accumulation of *gypsy* transcripts in permissive follicle cells is critical for proviral amplification because the resulting viral particles move from this somatic ovarian tissue to adjacent germ cells, leading to the integration of new proviruses into the germ line chromosomes of progeny (10). Not all strains contain such functional proviruses, but they all share defective elements inserted at the same location (pericentromeric heterochromatin of every chromosome) that are relics of very ancient integration events (27, 63). We previously reported circumstantial evidence that the *flamenco*-dependent *gypsy* repression involves a natural RNA-silencing mechanism that requires the Piwi Argonaute-like protein. Reporter constructs containing various sequences from within the *gypsy* 5'-untranslated region (5'UTR) can be repressed by this silencing mechanism, like functional *gypsy* proviruses (52). We have also found that restrictive ovaries contain 25- to 27-nt rasiRNA-like RNAs that are complementary to the *gypsy* 5'UTR RNA, supporting an RNA-silencing working hypothesis (52).

The purpose of the present study was to gain insights into the function of *Drosophila* rasiRNAs by Northern blotting analysis of the *gypsy* small RNAs. We show here that there is a strong bias for the antisense strand of these *gypsy* rasiRNAs and that their accumulation is correlated with the level of repression of their complementary targets. Such correlations point to the *gypsy* rasiRNAs as being the bona fide effectors of a repression that is specifically directed against sense *gypsy*

transcripts. This novel RNA-silencing mechanism, which operates in the somatic ovarian tissues, displays common characteristics (Dicer-2-independent biogenesis and 3'-end modification of the rasiRNAs, asymmetry in strand polarity and involvement of a member of the Piwi subclade of the Argonaute family of proteins) with the rasiRNA pathway that has just been reported to silence repetitive DNA in the gonads (51, 62).

MATERIALS AND METHODS

Fly care. Unless otherwise indicated, genetic materials and fly stocks are described in Flybase (<http://flybase.bio.indiana.edu>). Flies were grown at 25°C on standard *Drosophila* medium (17).

The permissive wOR(P) and restrictive wRev(R) strains were described previously (35). They are called empty strains because they are devoid of functional *gypsy* proviruses and only contain the defective heterochromatic proviruses found in all present-day flies (27, 63). In contrast, the A237 (unpublished) and MG1 (44) restrictive strains both contain, in addition, many functional *gypsy* proviruses, located on the X chromosome and the whole genome, respectively. The MG1(rec) stock was derived from MG1 as follows. After a series of eight successive backcrosses with attached-X females of an empty strain, the MG1 autosomes were replaced by autosomes devoid of functional *gypsy* proviruses. We checked that only the X chromosome of the backcrossed stock contained sequences able to be lighted up by fluorescence in situ hybridization (FISH) of a *gypsy* probe with salivary gland chromosomes. These X-linked proviruses were eliminated together with the associated *y v f mal* markers, by recombination of the *flam*^{MG1(R)} restrictive allele into the wOR(P) background.

The following mutant fly stocks were also used: *y w eyFlp*; FRT42D *der-2^{L81fsX}* (31), *w¹¹¹⁸;cn piwi¹/CyO* and *cn piwi³/CyO;ry⁵⁰⁶* (12), *aub^{HN2} cn bw/CyO* and *aub^{QC42} cn bw/CyO* (54), *w UAS-IIRacZ* (12A) (23), and *GMR-wIR* (30, 31).

Detection of small RNA species. The detection of small RNAs was performed essentially as described previously (52), with the following modifications. Total RNA was isolated from 100 pairs of ovaries by using TRIzol. The RNA was enriched for low-molecular-weight species by spinning for 20 min at 1,000 × *g* through Microcon YM-100 concentrators (Millipore). Alternatively, enrichment was performed by extracting the RNA with a mirVana kit (Ambion). About one-fourth of the ovarian extracts were loaded onto a 15% denaturing acrylamide-bisacrylamide (19:1) gel, transferred onto a Hybond NX membrane (Amersham), and fixed by UV cross-linking (0.5 J/cm²). Hybridizations with hydrolyzed RNA probes (50 nt, on average) were performed overnight at 40°C in 45% formamide–0.3 M NaCl–50 mM NaPO₄ (pH 6.8)–1× Denhardt solution–7% sodium dodecyl sulfate. Membranes were washed twice for 30 min at 60°C in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7])–0.5% sodium dodecyl sulfate and exposed for 2 days on a PhosphorImager screen (Molecular Dynamics). The sizes and specific radioactivities of both cRNA probes were compared by comigration on 8% denaturing acrylamide gels. The hybridization efficiencies were also calibrated by cohybridizing the Northern membranes with duplicate dots of dsRNA which was expected to be similarly lighted up by both RNA probes. To ensure that the RNA transcripts used as probes were either only sense or antisense, oligonucleotides corresponding to sense and antisense polarities were also spotted on these control membranes. After narrowing down the linear sensitivity range by using the “Gray/Color Adjust” function of ImageQuant, the exported tiff images were mounted with Adobe Photoshop without any further editing. The amount and integrity of the RNA were checked both by staining the gels with 2 mg of ethidium bromide/ml and by reprobating the unstripped membranes with an end-labeled oligonucleotide complementary to the mir-13b miRNA (5'-ACTCGTCAAAATGGCTG TGATA-3').

NaIO₄ reaction and β-elimination. This chemical reaction was performed essentially as described previously (1). Up to 20 µg of low-molecular-weight RNA was resuspended in 17.5 µl (30 mM borax, 30 mM boric acid [pH 9.0]). After the addition of 2.5 µl of freshly dissolved 200 mM NaIO₄, a 10-min incubation was performed in the dark at room temperature. To quench the unreacted NaIO₄, 2 µl of glycerol was added, followed by incubation for an additional 10 min in the dark at room temperature. Samples were almost completely dried by centrifugation under vacuum for 1 h at room temperature, resuspended in 50 µl of borax buffer (33.75 mM borax and 50 mM boric acid [pH 9.5] with NaOH), and incubated for 90 min at 50°C in dark. RNA was precipitated overnight at –20°C with 6 µl of 3 M sodium acetate and 168 µl of ethanol, collected by centrifugation, and then dissolved in the denaturing gel loading

buffer. As a control, the whole procedure was performed by replacing the NaIO_4 with water.

Plasmid construction and P element-mediated germ line transformation. As described previously (52), the pES2 and pES5 constructs (Fig. 1D) are carried by a transformation vector that includes the *white* gene as a selection marker. The parental pES2 plasmid consists of the *yp3* ovarian promoter, driving the expression of the *lacZ* reporter in the follicle cells (47). The 4342-4523 *gypsy* fragment was PCR amplified with primers containing *Sall* restriction sites at their non-*gypsy* 5' extensions. The pESpol- and pESpol+ plasmids were constructed by cloning this *Sall*-restricted 187-bp fragment into the 5'UTR of pES2 (XhoI restriction site) in the reverse and forward orientations, respectively.

P element-mediated transformation was performed as described previously (49). The constructs were coinjected with the pUChsΔ2-3 helper (a gift from D. C. Rio) into embryos of the permissive wOR(P) stock. Flies carrying the transgene were identified by rescue of the *white* phenotype. Inserts in transgenic flies were made homozygous in this *w flam^{OR(P)}* permissive background by endogamous crosses and selection for the most highly pigmented flies. The transformants were also backcrossed twice to wRev(R) females and then made homozygous in this restrictive background as well. Each pair of transgenes was analyzed (XbaI-restricted genomic DNA probed with the simian virus 40 trailer) to confirm 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–phosphate-buffered saline (PBS) for 5 min at room temperature, washed twice with PBS, rinsed once in staining buffer [1 mM MgCl_2 , 4 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 4 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 1% Triton X-100], and stained for 0.5 to 4 h at 37°C 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. Digital images were captured using the same settings and processed together after having been mounted in a single Adobe Photoshop file.

In situ hybridization. Detection of RNA in whole-mount ovaries and female larval gonads was essentially performed as previously described (41) with the following modifications. Glutaraldehyde, dimethyl sulfoxide, heparin, levamisole, and RNase treatments were omitted. Hybridization was performed on wandering female larvae that had been inverted after cutting off the head (the proteinase K treatment was omitted, too). The prehybridization and hybridization temperatures were 55 and 65°C, respectively. Digoxigenin-labeled RNA probes, a 1.2-kb *gypsy* antisense fragment (coordinates 1425 to 199), and a 2.5-kb *lacZ* antisense fragment (accession number J01636, coordinates 2100 to 4600) were not hydrolyzed. Washings were performed at 55°C in hybridization buffer and then in PBS containing 0.1% Tween 20. The background was destained by soaking overnight in 95% ethanol. Ovaries and larvae were stored in PBS–75% glycerol, dissected, mounted in PBS–90% glycerol, and viewed under a Leica DMRB microscope. Digital images were captured using the same settings and processed together after having been mounted in a single Adobe Photoshop file.

RNA secondary structure prediction. The sequence analysis was done online at <http://www.genebee.msu.su/genebee.html>. Each selected sequence, flanked by at least 100 nt at both ends, was checked for the absence of stems longer than 20 nt.

RESULTS

Unlike miRNAs, both *gypsy* rasiRNAs and hairpin-induced siRNAs are modified at their 3' termini. As reported recently (62), the 3' terminus of rasiRNAs respectively produced by the *Su(Ste)* and *roo* repeated genetic elements in the testis and ovary differ from those of the miR-8 and miR-311 microRNAs. The absence of either a 2' or a 3' hydroxyl group makes them resistant to the β-elimination reaction which, on the contrary, causes the loss of the terminal nucleoside of NaIO_4 -pretreated microRNAs. We repeatedly observed that the 24- to 29-nt rasiRNAs detected by riboprobes complementary to antisense *gypsy* sequences from within the long terminal repeat and *env* regions (Fig. 1C) were not affected by the NaIO_4 and β-elimination treatments (Fig. 2A, top, lanes 2 and 3). In contrast, in the very same samples, the miR-13b-positive control RNA

reacted to completion with periodate, as shown by the apparent 2-nt shift downward in mobility resulting from the loss of one nucleotide and the gain of one negative charge (Fig. 2A, bottom, compare lanes 2 and 3).

Unlike plant mi- and siRNAs (15, 32, 66), animal siRNAs are thought to have terminal hydroxyl groups at both the 2' and the 3' positions and to be therefore sensitive to β-elimination. However, we observed (Fig. 2B) that the 21-nt RNAs resulting from the expression in the somatic ovarian tissues of a *lacZ* hairpin construct known to trigger RNAi (23) did not react with NaIO_4 altogether. Similarly, we inferred from the result of this β-elimination assay that the 21-nt RNAs derived from the expression in the head of the *GMR-wIR* hairpin RNAi trigger (30, 31) also lack either or both of the 2' and 3' terminal hydroxyl groups (Fig. 2C). Modification of their 3' ends do not, however, prevent such hairpin-induced siRNAs from performing effective in vivo silencing as shown below (see Fig. 6 and 7). In this respect, they therefore look like plant mi- and siRNAs (15, 32, 66), as well as fly rasiRNAs.

Unlike siRNAs, *gypsy* rasiRNAs are strongly unbalanced in favor of the antisense strands. The presence of a small fraction of *gypsy* dsRNA in tissue cell culture total RNA was reported long ago (21). Like siRNAs and miRNAs, *Drosophila* rasiRNAs were assumed to be the products of RNase III processing of the dsRNA precursors (4). As a control, we first checked that both strands of the *GMR-wIR* hairpin RNA precursor were equally represented in the population of their siRNA products (Fig. 2C). We then wondered whether *gypsy* rasiRNAs would also contain both strands of the *gypsy* sequence. To address this question, we first used both strands of the 120-nt *gypsy* 5'UTR fragment as probes (Fig. 1C). Although sense-oriented RNAs with the same size as the antisense strands were indeed detected, they were notably less abundant than their antisense counterparts (data not shown). Three additional small fragments were then randomly chosen from the complete *gypsy* proviral sequence (accession number M12927, coordinates 2188 to 2368, 4342 to 4526, and 6541 to 6718) to produce ~180-nt strand-specific RNA probes (Fig. 1C).

The results obtained with these three *gypsy* fragments (Fig. 3) were in agreement with the observations made with the 120-nt fragment from within the *gypsy* 5'UTR. (i) We were able to detect small RNAs corresponding to each fragment, suggesting that most (if not all) *gypsy* sequences are likely to be represented in the population of *gypsy* rasiRNAs. (ii) Their sizes ranged from 23 to 29 nt. (iii) Both strands of the *gypsy* sequence could be detected, as if they were born double-stranded from long dsRNA precursor(s). (iv) However, regardless of the particular fragment used as a probe, the antisense *gypsy* rasiRNAs (Fig. 3A, B, E, F, I, and J) were always clearly more abundant than the corresponding sense strands (Fig. 3C, D, G, H, K, and L, respectively). An even greater, although also unexplained, imbalance has just been reported for the *Su(ste)* and *roo* rasiRNAs in *Drosophila* (62). In the other way, plants infected by positive RNA viruses have been reported to preferentially accumulate sense-stranded, hairpin-derived viral ds-siRNAs (37). A similar explanation for our results, which would assume that these mostly antisense *gypsy* rasiRNAs are processed from antisense *gypsy* hairpins, was ruled out by the following two observations: first, we could not predict any 20-nt hairpins for any of the three tested sequences (see Materials

FIG. 2. Anatomy and biogenesis of rasiRNAs, miRNAs, and siRNAs. (A) The *gypsy* rasiRNAs fail to react with periodate, and their production does not require the Dicer-2 RNase III nor a high *gypsy* copy number. Low-molecular-weight-enriched RNA extracted from ovaries, submitted or not to NaIO_4 treatment, was hybridized with a sense *gypsy* RNA probe (coordinates 6541 to 6718 in the *gypsy* sequence, accession number M12027) schematized in Fig. 1C. Hybridization of the membrane with the 310-433 sense riboprobe led to similar observations (data not shown). The names of the strains and/or genotypes used in this experiment are indicated above the corresponding lanes (see Materials and Methods for a description of the strains). The stripped membrane was reprobed with the end-labeled oligonucleotide complementary to the abundant 23-nt *mir-13b* miRNA (bottom panel). The amount of rasiRNAs in the MG1(rec) strain was found to be ~ 0.8 -fold that of the parental MG1 strain, after standardization with the miRNA loading control. A mixture of 5'- ^{32}P -radiolabeled 25- to 26-nt RNA oligonucleotides was added to the Decade kit (Ambion) to be used as size markers. At the top of the upper panel, the signal resulting from nonspecific hybridization with the abundant 30-nt 2S rRNA is the only one to be sensitive to the NaIO_4 treatment. (B) Unlike miRNAs, the 21-nt siRNAs triggered by *Actin5C-Gal4*-driven expression of the UAS-*IRlacZ* hairpin in the somatic ovarian tissues are resistant to the periodate treatment. Low-molecular-weight enriched RNA extracted from ovaries, subjected or not to NaIO_4 treatment, was hybridized with an antisense *lacZ* RNA probe (top panel) and reprobed with *mir-13b* (bottom panel). (C) In vivo expression of the *GMR-wIR* hairpin in the eye produce 21-nt siRNAs of both polarities that fail to react with periodate. Shown are two quantitative Northern blots of *mirVana*-extracted fly head RNA (Ambion). They were hybridized with either strand (s, sense; as, antisense) of a *white* exon 3 RNA probe. Proper standardization of both probes was achieved by cohybridization with dsRNA duplicate dot blots (not shown; see Materials and Methods). The bottom panels correspond to the simultaneous hybridization of both stripped membranes with the same *mir-13b* oligonucleotide probe. Different amounts of markers were loaded in lanes 5 and 6.

and Methods), and second, even if hairpins did exist, they would probably be mostly sense oriented, in view of the excess of sense *gypsy* transcripts (data not shown), which would therefore mostly produce sense rasiRNAs. (v) The three *gypsy* probes disclosed the interstrain variation in the amount of rasiRNAs previously observed with the 5'UTR RNA probe (52). Indeed, for each of the six strand-specific probes, the restrictive MG1 strain (Fig. 3B, C, F, G, J, and K) showed a significantly stronger signal than the restrictive wRev(R) strain (Fig. 3A, D, E, H, I, and L, respectively). In the latter strain, the amount of antisense rasiRNAs complementary to the 4342-4526 sense probe was so low that it was indistinguishable from that of the permissive wOR(P) strain (data not shown). If, as hypothesized earlier, these rasiRNAs are actually the effectors of *gypsy* regulation, then the strongest repression should occur against sense-oriented *gypsy* targets in the MG1 ovaries, whereas the 4342-4526 sense sequence should not be targeted by the repression in the wRev(R) strain.

The amounts of *gypsy* rasiRNAs are correlated with the sensitivity of the complementary targets to repression. As shown in Fig. 1D, the targets that we decided to test corre-

spond to both orientations of the small "*pol*" fragment that has been previously used to detect rasiRNAs. Four pESpol+ (sense orientation) and three pESpol- (antisense orientation) independent transgenic inserts were tested in three different genetic backgrounds, including the wOR(P) permissive strain and the F_1 progeny of crosses between the wRev(R) and MG1(R) restrictive strains. A sample of the results that were reproducibly obtained in three independent assays is shown in Fig. 4. In good correlation with the differential accumulation of the complementary rasiRNAs in the MG1(R) background (Fig. 3, lanes F versus G), all four pESpol+ transgenes were repressed (compare, for instance, Fig. 4A and C), whereas none of the three pESpol- transgenes was repressed (Fig. 4D and F). This correlation indicates that the antisense "*pol*" rasiRNAs are most likely the effectors which specifically guide presumptive repressor complex(es) toward the pESpol+ target.

Moreover, we observed an almost complete lack of repression of the pESpol+ target in the wRev(R) restrictive background (Fig. 4A and B). This observation is in good agreement with the low abundance of the complementary rasiRNAs in

FIG. 3. Evidence for the overaccumulation of *gypsy* rasiRNAs antisense strands. Low-molecular-weight enriched RNA was extracted from whole ovaries of the wRev(R) and MG1 restrictive strains (see Materials and Methods for a description of the strains). Three pairs of duplicate Northern blots are shown. They were hybridized with either strand (s, sense; as, antisense) of three RNA probes that are schematized in Fig. 1C. Proper calibration of the pairs of RNA probes was achieved a posteriori by adjusting the Hi display levels of the ImageQuant files (see Materials and Methods) to obtain the same signal intensity on both dsRNA duplicate dot blots that were cohybridized with the Northern blots (not shown). The amount and integrity of the low-molecular-weight RNA loaded in each lane were checked by reprobing the unstripped membranes with the end-labeled oligonucleotide complementary to the abundant mir-13b microRNA (lower panels). The position of the mir-13b 23-nt major band is indicated by black squares. The amounts of *gypsy* rasiRNAs were quantified with ImageQuant software (Molecular Dynamics) and standardized using those of mir-13 as loading controls and those of the dsRNA dots as probe controls. The amounts of the antisense strands were always three- to fivefold in excess over those of the sense strands, whatever the strain and the fragment used as a probe.

this strain (Fig. 3E). The ability of this strain to repress transcripts of the *gypsy* retrovirus may be explained by the accumulation of antisense rasiRNAs homologous to other parts of the element, such as, for instance, the 5'UTR (52).

So, the fact that both the strain- and strand-specific variations in the amount of *gypsy* rasiRNAs are correlated with the sensitivity of the complementary targets strongly suggests that they are bona fide effectors of this silencing phenomenon.

Unlike transgene-induced silencing, the efficiency of *gypsy* silencing is not copy number dependent. In *Drosophila*, the posttranscriptional silencing of the endogenous *adh* gene depends both on the number and on the expression level of homologous transgenes, while the accumulation of complementary siRNAs varies accordingly (39). The high accumulation of *gypsy* rasiRNAs found in MG1 might result from the fact that this restrictive strain contains the highest copy number (about 30) of *gypsy* proviruses recently inserted into the euchromatic part of the genome.

To determine whether these proviruses do contribute to the production of *gypsy* rasiRNAs, we managed to get rid of them by inter- and intrachromosomal recombination (see Materials and Methods). Essentially, the pericentric region of the MG1 X chromosome, which contains a *flam* restrictive allele, was recombined into a permissive genome devoid of euchromatic proviruses. As a control, we then checked by FISH that the polytene chromosome arms of the newly obtained MG1(rec) strain did not contain any of the parental proviruses (data not shown). The fact that the amounts of *gypsy* rasiRNAs do not significantly differ between both strains (Fig. 2A, lanes 1 and 2, and data not shown) shows that they are not produced by euchromatic proviruses in a copy-dependent manner. This indicates that the bulk of *gypsy* rasiRNAs are produced by pericentromeric proviruses, even in a *gypsy*-rich strain such as MG1.

This mapping to the *flam* region of the genetic determinant of the wealth of MG1 rasiRNAs does not imply that the pericentromeric trigger(s) must also map there. We can imagine that some provirus(s) located in the autosomal heterochromatin of the permissive parental strain could become very

FIG. 4. *flam*-dependent downregulation of transcripts containing the 180-nt sense "pol" target. The two reporter constructs described in Fig. 1D, pESpol+ and pESpol-, were introduced by transgenesis into the permissive wOR(P) strain. Several transgenic inserts were then genetically combined with the *flamenco* genotypes indicated at the top. Their expression was monitored by *lacZ* staining of whole ovaries. Whole-mount in situ hybridization of ovaries was also performed with a *lacZ* RNA probe leading to similar observations (not shown). Significant repression was only observed in the MG1(R)/wRev(R) restrictive background and for the four ESpol+ transgenes, whose target is transcribed in the sense orientation (see panel C). Expression of the three ESpol- transgenes did not show any reduction in this restrictive genotype (see panel F). Only the results obtained with two transgenic inserts (F23 and M40) are shown here as examples.

efficient triggers when combined with an exceptionally efficient MG1 *flam* restrictive allele. So, the reason why the production of *gypsy* rasiRNAs by some pericentromeric trigger(s) is so efficient in this particular genetic background is still unknown.

Effect of RNA silencing mutations on the production of *gypsy* rasiRNAs and on the repression of *gypsy*. Accumulation of the *GMR-wIR* siRNAs is strongly reduced in *dcr-2* mutant *Drosophila* heads because they lack the Dcr-2 RNase III activity (31). To test the involvement of this RNAi gene in the repression of *gypsy*, we introduced the *dcr-2^{L811fsX}* recessive mutant allele into the *gypsy*-containing restrictive background of the A237 strain. *gypsy* rasiRNAs could still be detected at the same level in both the wild-type and the mutant *dcr-2* genotypes (Fig. 2A, lanes 4 and 5, respectively) showing that the Dcr-2 RNAi protein is not required for the production of these small RNAs. In order to check that the *dcr-2* allele used in this experiment was actually lacking the Dcr-2 function, we tested it with an RNAi-based assay directed against the endogenous *white* gene. In a wild-type context, a single dose of the *GMR-wIR* transgene can almost completely repress the *white* gene, producing a yellowish eye phenotype (data not shown). In contrast, in homozygous *dcr-2^{L811fsX}* mutant flies, we obtained a red-eyed phenotype, indicating a defective RNAi pathway (see Fig. 6C).

In the RNAi silencing pathway, Dcr-2 is not only essential to dice dsRNAs into smaller siRNAs but also to assemble the silencing complex (43). That is why *dcr-2* mutants are only partially rescued by the exogenous supply of siRNAs that should bypass the first deficient dicing step (31). To test whether Dcr-2 is similarly involved in the silencing of *gypsy* downstream of the rasiRNA production, we monitored the expression of the *gypsy* proviruses that were introduced into the mutant stock by the X restrictive chromosome of the A237 strain. Whole-mount in situ hybridization of an antisense *gypsy* riboprobe with either larval female gonads or adult ovaries revealed that these proviruses were still repressed in the *dcr-2* mutant background (Fig. 5A2 and B2).

As reported previously, a *gypsy-lacZ* reporter is derepressed in the larval female gonads when introduced into a *flam^R;piwi²* genetic background (52). We checked that the *piwi¹* and *piwi³* mutant alleles also knocked down the repression of the endogenous *gypsy* proviruses in the same conditions (Fig. 5A4 and A6). In contrast, *aub*, the *piwi* paralog, is not required for *gypsy* silencing (Fig. 5A8 and B4).

***Piwi*, *aub*, and *flam* are not required for RNAi in vivo.** In the *GMR-wIR* RNAi assay described above, *white* was not derepressed in *piwi¹* and *piwi³* homozygous mutant flies (Fig. 6A and B), unlike in mutants for *dcr-2*, the typical RNAi gene (Fig. 6C). So, in the eye, *Piwi* is required for both cosuppression (39) and *PcG*-dependent repression (19) but not for hairpin-induced RNAi. Similarly, *GMR-wIR* could still repress *white* in *aub^{N11}/aub^{HN2}* (Fig. 6D) and *aub^{N11}/aub^{QC42}* eyes (data not shown), whereas these mutant genotypes are known to impair *PcG*-dependent repression in the eye (19), as well as RNAi induced by the injection of dsRNA into embryos (24).

To test the effect of *flam* on RNAi, we had to switch to another RNAi assay that could be performed in the follicle cells where *flam* is known to be expressed. The ES2 and ES5 constructs (Fig. 1D), known to be expressed in these somatic ovarian tissues (52), were used as RNAi targets. As previously reported, the ES2 transgene, which does not contain *gypsy*

FIG. 5. Effect of three RNA silencing genes on the *flam*-dependent repression of *gypsy*. Whole-mount in situ hybridization was performed with a digoxigenin-labeled antisense *gypsy* probe (coordinates 1425 to 199) on either larval female gonads (A and C1) or adult ovaries (B and C2). Larval gonads were oriented so that the anterior is to the top. In panel C1, the magnification is twice the magnification in panel A. Only the ovarioles that contain a stage 10 egg chamber were dissected from the ovaries. Except for a *gypsy*-containing permissive control strain (C), all genotypes were homozygous for the same *gypsy*-containing restrictive X chromosome. Mutant genotypes (*m/m* = even numbers) were sorted from the corresponding control heterozygous sisters (*m/+* = odd numbers) using the dominant phenotypes of the *Actin5C-GFP* and *Cy* markers present on the balancer chromosome. The *dcr-2^{L811fsX}* (A2 and B2), *piwi¹* (A4), and *piwi³* (A6) alleles were tested as homozygous, whereas transheterozygous ovaries, *aub^{QC42}/aub^{HN2}* (A8 and B4) were obtained. Significant derepression of the endogenous *gypsy* proviruses was only observed in the *piwi¹* and *piwi³* homozygous larval female gonads. The reason why gonads were more labeled in A7 and A8 than in A1, A2, A3, and A5 is because, in the *aub* experiment, the substrate was incubated longer than in the other series of experiments. An additional positive control for a high sensitivity of the digoxigenin detection in this experiment is provided by the strong staining of the gonad-associated fat body (A8, arrowheads), where *gypsy* is moderately expressed. All adult ovaries exhibited the same restrictive phenotype, consisting in a weak signal restricted to the centripetal and nurse-cell-associated follicle cells.

sequences, is not sensitive to *flam*-dependent repression (Fig. 7A and E), while ES5, which contains a 59-nt *gypsy* fragment, is repressed in the *flam* restrictive background (Fig. 7C and G). A *lacZ* hairpin was expressed in the somatic ovarian tissues by

FIG. 6. *piwi* and *aub* are not required for hairpin-induced RNAi in the eye. Shown are typical examples of the level of *white* repression by the *GMR-wIR* transgene in the indicated mutant backgrounds. Targeting the *white* gene by RNAi resulted in a very penetrant yellowish eye color. On the grayscale picture, this repression appears as a much lighter gray (see panels A, B, and D) than the dark gray corresponding to the reproducible reddish eye color seen in the *dcr-2*-null background (see panel C).

the UAS*IRlacZ* transgene (23) under control of the *Act5C-GAL4* driver. The hairpin could target the homologous *lacZ* region of the reporter constructs not only in the restrictive background (Fig. 7B) but also in the absence of any *flam* repressive activity (Fig. 7F and H). Thus, the Flamenco function that is involved in the RNA silencing of *gypsy* in the ovary is not required for RNAi in this tissue.

DISCUSSION

gypsy rasiRNAs appear to be the bona fide effectors of a RNA silencing mechanism directed against sense *gypsy* transcripts. Expression of the *Ste* and *roo* repeated genetic elements is increased in RNA silencing mutant strains showing reduced accumulation of the corresponding rasiRNAs (5, 7, 62). Similarly, in the present study, we provide correlations between the silencing of another element, *gypsy*, and the presence of complementary rasiRNAs. First, the highest accumulation of these small RNAs was observed precisely where the strongest repression occurred, i.e., in the ovaries of the MG1 strong restrictive strain. Second, an asymmetry in rasiRNA strand polarity (in favor of antisense strands) was correlated with a preferential sensitivity of the (sense) complementary targets.

Thus, the fact that the *yp3-lacZ* heterologous transcription unit could be made sensitive to the repression when fused with the small “*pol*” fragment in the sense, but not antisense, orientation confirms previous hints (52) that the prevalent antisense *gypsy* rasiRNAs cannot target *gypsy* at the DNA but at the RNA level. Note, however, that this does not rule out a model of transcriptional silencing that would be targeted via the hybridization of rasiRNAs with nascent transcripts.

Genome integrity is maintained by similar rasiRNA-mediated silencing pathways operating either in the somatic or germinal lineages. Most transposable elements have to be expressed in the germ line to increase their copy number by transposition. However, some endogenous retroviruses escape this germ line tissue specificity constraint because they can invade the germ line from the permissive follicle cells where they are expressed. Indeed, the amplification of *gypsy* and *ZAM* proviruses involves expression and assembly of particles in this somatic tissue, transfer to the germ line and integration in the progeny (9, 10). It is also known that transfer to the germ line

FIG. 7. *flam* is not required for hairpin-induced RNAi in the somatic ovarian tissues. Histochemical staining of the β -galactosidase activity in follicle cells containing either of the ES2 and ES5 transgenic reporters (see Fig. 1D for a description of the constructs). Expression of the *lacZ* transgene in the follicular epithelium is detected by a dark-blue staining of the X-Gal substrate that appears in black on the grayscale pictures. The presence in the ES5 transgenes of a 59-nt *gypsy* fragment is known to make them sensitive to the restrictive *flam* activity (compare panel C with panel G). The UAS*IRlacZ* hairpin was expressed in the somatic ovarian tissues by virtue of the activation of its somatic UAS promoter by the ubiquitous *Actin5C-Gal4* driver. This resulted in a total RNAi-mediated *lacZ* repression not only in the presence (B) but also in the absence (F and H) of any *flam* activity.

(endogenization) of *gypsy* is highly efficient after infection (25, 58). The novel rasiRNA-mediated silencing phenomenon that we describe here definitely operates in these ovarian somatic tissues. By virtue of its tissue specificity, *gypsy* silencing can therefore protect the *Drosophila* genome from both the expansion of endogenous proviruses and the endogenization process of viruses transmitted horizontally.

A similar RNA-silencing mechanism has just been shown to protect the *Drosophila* genome from the deleterious expression of repeated elements in the germ line (62). So, both the germinal and the somatic lineages appear to use very similar rasiRNA-mediated silencing mechanisms that display the following differences with the miRNA- and/or the siRNA-dependent silencing pathways. Unlike miRNAs, the 3' ends of rasiRNAs and siRNAs have 3'-end modifications that make them resistant to the β -elimination reaction. RNAi involves the loading of siRNAs by the AGO2 slicer protein. In contrast,

the AGO-like Piwi protein is required for *gypsy* silencing in the larval female gonads, while Piwi (as well as its paralog, the Aub protein) was reported to specifically interact with various rasiRNAs in the ovary (51, 62). Moreover, we show here that neither Piwi nor Aub are required for RNAi, at least in the eye, another imaginal somatic tissue.

Biogenesis of *gypsy* rasiRNAs. The biogenesis of rasiRNAs also seems to be quite different from that of other effectors of RNA silencing. Like the *roo* and *Su(Ste)* rasiRNAs, the *gypsy* rasiRNAs are longer (24 to 30 nt) than miRNAs, siRNAs and any small RNA ever characterized biochemically as products of the three *Drosophila* RNase III enzymes (8, 33, 50). As expected, their accumulation was found to be independent of the RNAi-specific Dcr-2 RNase III activity. Moreover, the aforementioned strong asymmetry in strand polarity sheds further doubts about the existence of a dsRNA precursor.

The *Su(Ste)* rasiRNAs seem indeed to be exclusively of the antisense polarity (62). The complete absence of one strand is also typical of piRNAs, the small RNAs recently reported to interact with Piwi-like proteins in the testes of mammals (3, 18, 28). Concerning the *roo* retroelement, a few sense rasiRNAs were observed but, unlike antisense *roo* rasiRNAs, they were not affected in *armi* mutant ovaries, in which *roo* expression is increased, suggesting that they do not contribute to *roo* silencing (62). Similarly, a low level of sense *gypsy* small RNAs was detected in our Northern blots, but these RNAs were not able to silence the complementary target. So, the few *roo* and *gypsy* small sense RNAs might not be bona fide rasiRNAs and all *Drosophila* rasiRNAs would be exclusively of the antisense polarity. The mechanism of production (i.e., what precursor is used by what enzyme?) of such predominantly antisense rasiRNAs is nevertheless still elusive.

The rasiRNAs production is triggered by some pericentromeric *gypsy* provirus(es). The pericentric *flam* region of the strongest restrictive MG1 strain was recombined into a permissive genome devoid of euchromatic proviruses, and the recombinant strain turned out to contain as many *gypsy* rasiRNAs as the parental MG1 strain. The results of this genetic recombination experiment indicate that the genetic determinant(s) responsible for the unusually high level of *gypsy* rasiRNAs in the MG1 strain map at the base of the X chromosome where *flam* is located. This provides additional evidence that *flam* is involved in the production and/or stabilization of *gypsy* rasiRNAs (52). However, since *flam* has not yet been molecularly characterized (46), we cannot choose between the two following hypotheses. (i) Putative *gypsy* provirus(es) inserted in this repetitive DNA-rich region of the X restrictive chromosomes might be expressed in such a way (i.e., aberrant transcription from flanking repetitive sequences?) as to trigger the production of rasiRNAs. This model is reminiscent of that of the 1A region, rich in telomeric associated sequences, apparently responsible for the *trans*-silencing, in the *Drosophila* female germ line, of any target homologous to elements inserted therein (48). (ii) Instead of *gypsy* provirus(es) inserted at the base of the X chromosome, *flam* might be a heterochromatic RNA silencing gene involved in the production or stabilization of rasiRNAs triggered by proviruses inserted elsewhere in the genome. Concerning other retroelements, it would be interesting to know whether *flam* is also able to control the accumulation of the corresponding rasiRNAs in

the ovary. With regard to siRNAs, *flam* is probably not involved in their accumulation since we show here that hairpin-induced RNAi can be observed in the ovary independently of the *flam* genetic background.

Even in the second model, not all *gypsy* proviruses can drive the production of rasiRNAs. Indeed, the amount of rasiRNAs was not reduced in the recombinant strain, even though the permissive genome into which the strong MG1 *flam* restrictive allele was recombined is devoid of functional *gypsy* proviruses and only contains defective pericentromeric *gypsy* insertions. Thus, the many functional euchromatic proviruses present in the MG1 genome do not contribute significantly to the bulk of rasiRNAs. In this model, *gypsy* rasiRNAs would be triggered by some pericentromeric (not necessarily X-linked) defective provirus(es) shared by all *Drosophila* strains, including the permissive parental strain, under control of the *flam* restrictive function.

In both models, only pericentromeric endogenous provirus(es) would be able to trigger preprogrammed, RNA-mediated resistance of their host to the cognate functional retroviruses. Such an innate immunity system would compensate for the inability of retroviruses to trigger RNAi because of the lack of dsRNA during retroviral replication. Similarly, the repression by the host of many other transposable elements might also take advantage of the presence of defective copies of such elements in heterochromatin. These copies would be maintained not only because they are less deleterious in this gene-poor region of the genome but also because they would build up a repertoire of rasiRNA-producing sequences subsequently used by similar innate immunity systems to ensure genomic stability.

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