piRNA-mediated nuclear accumulation of retrotransposon transcripts in the *Drosophila* female germline

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Germline silencing of transposable elements is essential for the maintenance of genome integrity. Recent results indicate that this repression is largely achieved through a RNA silencing pathway that involves Piwi-interacting RNAs (piRNAs). However the repressive mechanisms are not well understood. To address this question, we used the possibility to disrupt the repression of the *Drosophila* I element retrotransposon by hybrid dysgenesis. We show here that the repression of the functional I elements that are located in euchromatin requires proteins of the piRNA pathway, and that the amount of ovarian I element piRNAs correlates with the strength of the repression in the female germline. Antisense RNAs, which are likely used to produce antisense piRNAs, are transcribed by heterochromatic defective I elements, but efficient production of these antisense small RNAs requires the presence in the genome of euchromatic functional I elements. Finally, we demonstrate that the piRNA-induced silencing of the functional I elements is at least partially posttranscriptional. In a repressive background, these elements are still transcribed, but some of their sense transcripts are kept in nurse cell nuclear foci together with those of the Doc retrotransposon. In the absence of I element piRNAs, either in dysgenic females or in mutants of the piRNA silencing pathway, sense I element transcripts are transported toward the oocyte where retrotransposition occurs. Our results indicate that piRNAs are involved in a posttranscriptional gene-silencing mechanism resulting in RNA nuclear accumulation.

hybrid dysgenesis | I element | RNA silencing

A large fraction of eukaryotic genomes is made of transposable elements. Their mobilization at high frequency is deleterious, resulting in high rates of mutations, chromosomal rearrangements, sterility, and a variety of other disorders. Mechanisms that repress transposition and keep it at levels compatible with species survival have been selected during evolution. These mechanisms have been a mystery for a long time. It is only recently that RNA silencing was shown to play an essential role in the control of transposable elements (see ref. 1 for a review).

RNA silencing collectively refers to a series of small RNA-mediated mechanisms controlling a large variety of biological processes, such as development, genome stability, and spreading of viruses and transposable elements. In *Drosophila*, four RNA silencing pathways have so far been distinguished but they may sometimes overlap. The processing of various double-stranded RNA precursors can produce three classes of 21 to 23-nucleotide RNAs, called microRNAs (miRNAs) and either exogenous (exo-) or endogenous (endo-) small interfering RNAs (siRNAs). The fourth class consists of Piwi-interacting RNAs (piRNAs), also called repeat-associated siRNAs (rasiRNAs). They are longer than the small RNAs of the other three classes and range from 24 to 29 nucleotides. Because most endo-siRNAs and piRNAs are complementary to repeated sequences, they are potentially able to repress transposition.

piRNAs appear to be specifically bound to either of the three proteins (Piwi, Aub, and Ago3) of the Piwi clade of the Argonaute family (2, 3). In the *Drosophila* ovary, Piwi is localized in the nucleus of germ and somatic cells (4). Aub and Ago3 are expressed essentially in the germline and accumulate in a cytoplasmic perinuclear structure of the nurse cells, called the nuage (2, 3). *Drosophila* piRNAs associated with Piwi and Aub are mostly derived from antisense strands of transposable elements (2, 5–7), while Ago3 is associated preferentially with piRNAs of sense polarity (2, 3). It has been shown that antisense piRNAs are produced by heterochromatic loci that transcribe clusters of transposable element remnants in antisense orientation (2, 8, 9). Sequencing of small RNAs suggests that Piwi- and Aub-associated antisense piRNAs might generate sense piRNAs by targeting the transcripts of euchromatic functional elements. Sense piRNAs might be used in turn to produce additional antisense piRNAs by guiding the cleavage of the antisense transcripts synthesized by the heterochromatic loci (2, 3). This hypothetical amplification cycle, known as the "ping-pong" model (2), might boost a sort of innate immunity against the activity of transposable elements.

The mechanisms by which piRNAs control the expression of transposable elements are not well characterized. One main question is to determine whether they undergo transcriptional or posttranscriptional silencing. Recent results lead to the conclusion that it is transcriptional (10). However, the available data on the silencing of transposable elements are often difficult to discuss because it is usually not known whether functional or defective elements were monitored. Moreover, most of the molecular characterizations of small RNAs in *Drosophila* were performed with whole ovaries that are a mixture of somatic and germ cells, which did not discriminate between phenomena occurring specifically in each cell lineage. Elucidation of the mechanisms requires focusing on fully characterized copies of transposable elements, the expression and transposition of which can be experimentally controlled in a given cell lineage.

The study of the I element fulfills these requirements. Indeed, this *Drosophila* retrotransposon is derepressed specifically in the germline when introduced into a naive genome (devoid of functional copies of the element; that is, elements that are able to transpose), which results in the so-called I-R hybrid dysgenesis syndrome that includes high rates of transposition and a correl-

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ative decrease of female fertility. The *Drosophila* I retrotransposon belongs to the class of non-long-terminal repeat retrotransposons that includes mammalian L1 elements. It is 5.4-kbs long and ends at the 3′ end by several TAA repeats. It contains two large ORFs, one of which (ORF2) exhibits nuclease, reverse transcriptase, and RNase H domains. It is transcribed under the control of a female germline-specific internal promoter (see refs. 11–13 for reviews).

There are two categories of strains relative to the I element: reactive (R) that are devoid of functional I elements, and inducer (I), the genome of which contains about 10 copies of the I element dispersed in euchromatin. Both categories of strains possess defective I elements located in pericentromeric heterochromatin (14). In I strains, I elements are silenced and do not transpose. By contrast, crosses between R and I strains produce dysgenic females in the germline of which I elements are no more repressed and transpose at high frequency. They do not transpose in males nor in somatic tissues of dysgenic females. Functional I elements are transcribed in the nurse cells of dysgenic ovaries, producing a full-length transcript that is used as both a retrotransposition intermediate and a messenger RNA for synthesis of the proteins required for transposition (15). This transcript is transported into the oocyte, where it accumulates in the vicinity of the nucleus where retrotransposition occurs. This requires both an RNA localization signal located in the middle of ORF2, which consists in a small stem loop (16), and the protein produced by ORF1 (17).

There is a maternal contribution in the control of I elements. This is illustrated by the strong difference observed in the amounts of transcripts and transposition frequencies of I elements between the isogenic females, resulting from reciprocal crosses between R and I strains: I element transcripts are more abundant and retrotransposition occurs at a much higher frequency in the dysgenic females, known as SF females, originating from crosses between R females and I males, than in the isogenic dysgenic females, called RSF females, coming from crosses between I females and R males. This suggests that repressor molecules are transmitted from I strain females to their progeny (15, 17).

We report here experiments showing that down-regulation of the expression of functional I elements does not require proteins involved in the miRNA and siRNA pathways. Instead, we show that it is achieved by typical effector proteins of the piRNA pathway and is quantitatively correlated with the accumulation of piRNAs. The production of piRNAs requires the presence of functional euchromatic I elements. This is biological evidence supporting a hypothesis, according to which the transcripts of a functional transposable element are required for the detectable production of antisense piRNAs by complementary heterochromatic defective elements. Finally, we demonstrate that this piRNA-mediated silencing of euchromatic functional I elements in the female germline is, at least in part, posttranscriptional. Functional I elements are transcribed, but some of the transcripts of the repressed I elements in I strains are kept inside the nurse cell nuclei in a piRNA-dependent manner. They accumulate in nuclear foci in which the transcripts of other transposable elements colocalize.

Results

Proteins Involved in the piRNA Pathway Are Required for I Element Silencing. *In situ* hybridization experiments have shown that I element transcripts are produced in the nurse cells of dysgenic females and are transported to the vicinity of the oocyte nucleus. These transcripts cannot be detected in the oocytes of I females, nor in the female somatic tissues, nor in the males (15, 17) (see also Fig. 2*B*).

To test the requirement of candidate genes for the silencing of I elements in the female germline, we performed *in situ* hybridization experiments on whole-mount ovaries of I females carrying various mutations for genes required for one or another of the four RNA silencing pathways. We checked that all of the stocks used in this analysis were I and looked for the potential derepression of functional I elements by the accumulation of I transcripts in the oocyte.

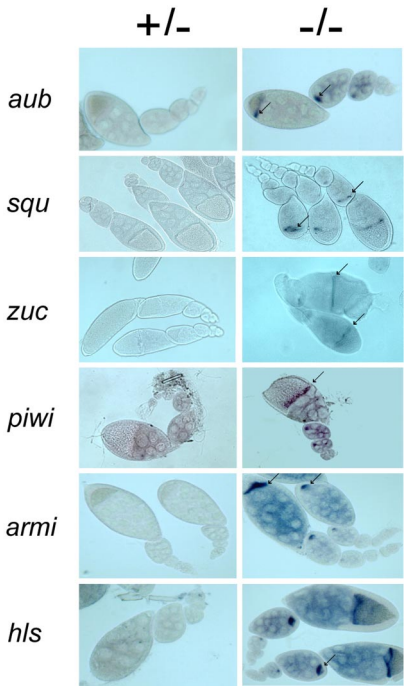

Fig. 1. Most of the genes whose mutations derepress I elements belong the piRNA pathway. *In situ* hybridization on whole-mount ovaries was performed using the 186-base pair long digoxigenin-labeled I element PCR product as a probe. The expression of the I elements was compared between control heterozygous ovaries denoted (+/−) and their siblings denoted (−/−). The latter were either homozygous (*piwi*^{1}) or transheterozygous (*aub*^{QC42}/*aub*^{N11}, *squ*^{HE47}/*squ*^{PP32}, *zuc*^{HM27}/*zuc*^{SG63}, *armi*$^{72.1}$/*armi*^{1}, *hls*^{E616}/*hls*^{3987}). *piwi*^{1} ovaries were obtained as germinal clones (see *SI Text*). Two independent experiments were performed for each mutant. Derepression of the I elements was observed in every mutant as illustrated by the accumulation of I element transcripts in the oocyte (*arrows*).

We first showed that genes like *dcr1*, *dcr2*, and *ago2* involved in the miRNA and siRNA pathways are not required for I element silencing [supporting information (SI) Fig. S1].

We then studied genes known to be involved in the piRNA-induced silencing. We confirmed previous data (18) indicating that I elements are up-regulated in *aubergine* (*aub*) mutants, and extended this observation to *squash* (*squ*) and *zucchini* (*zuc*) (Fig. 1). Because the somatic function of Piwi is required for the continuous production of egg chambers, we analyzed the effect of *piwi* by producing germinal clones of *piwi*^{1} (see *SI Text*) and observed that I elements were expressed in 10% ovarioles (see Fig. 1). These results show that all of the genes of the piRNA pathway that we have studied are required for I element silencing. Most of them encode proteins that localize in the nuage (19). We also tested some genes that do not clearly discriminate this pathway. The *Rm62* (*Lip*) gene was not found to be required for I element repression (see Fig. S1) while others, like *armitage* (*armi*) and *homeless*/*SpindleE* (*hls*/*SpnE*), were (see Fig. 1).

Euchromatic I Elements Trigger the Production of piRNAs Whose Accumulation Correlates with the Level of I Element Repression. The piRNA silencing pathway is characterized by the production of 24 to 29 nucleotide RNAs complementary to both strands of

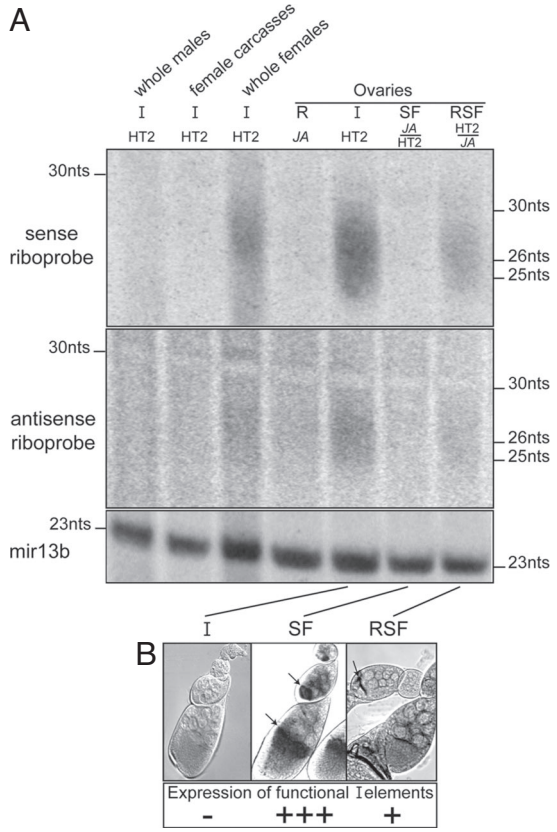

Fig. 2. The presence of functional I elements triggers the production of I element piRNAs in amounts that correlate with their repression level. (*A*) Northern blot hybridized successively with a sense I element riboprobe (*Top*), an antisense I element riboprobe (*Middle*), and a mir13b oligonucleotide probe (*Bottom*). The 23-nucleotide long mir13b miRNA was used as a loading control. RNAs enriched in low molecular weight RNAs were extracted from whole males, whole females, and female carcasses of the *HT2* (I) strain, and from ovaries of the *JA* (R) and *HT2* (I) isogenic strains and of the SF and RSF females generated by crossing *JA* females to *HT2* males and *HT2* females to *JA* males, respectively. (*B*) *In situ* hybridization on whole-mount ovaries was performed using the 186-base pair long-PCR product of the I element as a probe. Arrows show the accumulation of I element transcripts in the oocyte. The lower panel gives a qualitative assessment of these expression levels. I, SF and RSF ovaries are the same as in (*A*).

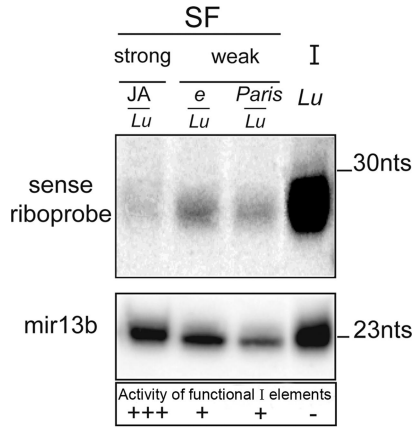

Fig. 3. The accumulation of I element piRNAs in dysgenic SF females is modulated by the reactivity level of their mothers. The upper panel corresponds to a Northern blot of low molecular weight ovarian RNAs probed with a sense riboprobe of the I element, as in Fig. 2*A*. The bottom panel shows the stripped membrane hybridized with the 23-nucleotide long mir13b oligonucleotide probe used as a loading control. Males of the standard I strain *Lu* were used to produce SF females by crossing with females of either *JA,* a strong R strain, or *e* and *Paris,* two weak R strains. The lower panel indicates the activity of the functional I elements that was estimated by measuring the intensity of the sterility of the females of each genotype (see *SI Text*, Drosophila *Strains and Fly Care*), which correlates with the frequency of transposition and the amount of transcripts of the I elements in their ovaries (12).

repetitive sequences. By Northern blotting, we looked for I element piRNAs in isogenic flies that differed either by the presence or absence of functional I elements dispersed in euchromatin [*HT2* (I) and *JA* (R) strains, respectively] or by the maternal origin [SF and RSF females produced by the reciprocal crosses between the *JA* (R) and *HT2* (I) strains]. RNAs were extracted either from whole flies or from ovaries or from female carcasses (the whole body, minus the ovary). In the *HT2* I strain we detected piRNAs of both polarities (although antisense look more abundant than sense piRNAs) in whole females and their ovaries but not in female carcasses nor in males (Fig. 2*A*). These piRNAs might therefore be present only in the *Drosophila* female germline where the I element is regulated.

Even more importantly, no piRNA of either polarity could be detected in *JA* R females, despite the fact that they are quite isogenic to the piRNA-rich *HT2* I females, except for the presence of functional I elements in the latter. Indeed, the former had been used as a recipient strain to obtain the latter by the introduction of a transgene containing a tagged I element. By subsequent replicative transposition, the euchromatic I element number had increased up to seven copies before the silencing became complete in this new I strain (20). This means that the mere addition of functional I elements into a naive R genome is sufficient to trigger the accumulation of piRNAs that will, in turn, repress these I elements.

piRNAs were also detected in SF and RSF ovaries (Figs. 2*A* and 3). Interestingly, their amount was higher in RSF females [issued from piRNA-rich *HT2* (I) mothers] than in isogenic SF females [issued from *JA* (R) mothers in which no piRNAs could be observed] (see Fig. 2*A*). Their accumulation is inversely correlated with the accumulation of I element transcripts in the oocyte (see Fig. 2*B*): I element transcripts were not detectable in I strain ovaries where I element piRNAs were the most abundant; they accumulated in SF ovaries where the amount of piRNAs was low, and intermediate amounts of transcripts were observed in RSF ovaries where piRNAs were present at intermediate levels (see Fig. 2). All these observations strongly suggest that the silencing of I elements in the ovarian germ cells is mediated by piRNAs, the accumulation of which depends on the presence of functional I elements in the euchromatin, and is also correlated with the amount of piRNAs present in the ovaries of the mother.

R strains are not identical in their permissivity, known as reactivity, to I element expression. Indeed, after crossing with standard I males, females from strains known as "strong R" strains produce SF females in which I elements transpose at very high frequency, while females of other R strains, known as "weak R" strains, produce SF females in which they transpose at a lower level. Reactivity displays an idiosyncratic maternal transmission (11–13). We showed that, although we could not detect I element piRNAs in R strains, whatever the reactivity level (Fig. S2), small RNAs were detected in SF females in amounts correlating with the ability of their R mothers to repress I element transposition (see Fig. 3). This provides an additional correlation between the level of repression of I elements and the amount of piRNAs accumulated in the ovaries.

The fact that we could not detect I element piRNAs in R ovaries by Northern blotting (see Fig. S2) does not mean that they are totally absent from R strains. A few I element piRNAs

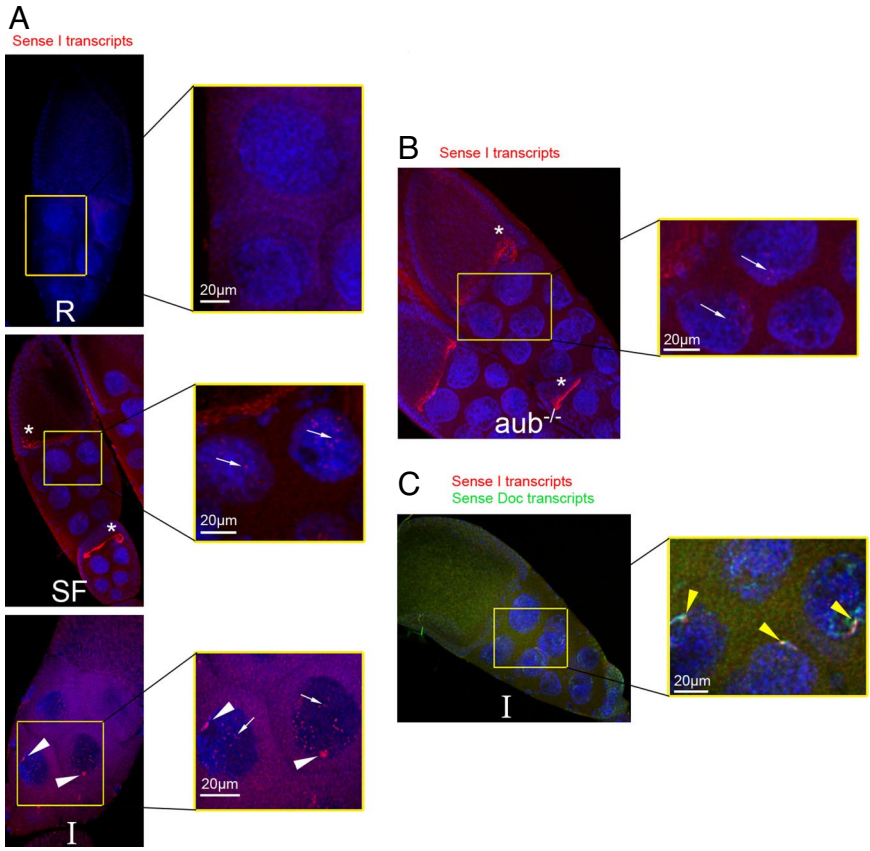

Fig. 4. RNA FISH on whole mount ovaries. (*A* and *B*) The ovaries originated from the *JA* (R) and *HT2* (I) strains, from SF females (SF) generated by crossing *JA* females with *HT2* males (*A*), and from mutant aub^{QC42}/aub^{N11} females ($aub^{-/-}$) (*B*). I element sense transcripts were detected in red by a rhodamine-conjugated antibody against a digoxigenin-labeled antisense riboprobe. Ovaries were counterstained with DAPI (*blue*). The white arrows (SF, I, $aub^{-/-}$) show the presence of putative nascent sense I element transcripts; the white arrowheads (I) indicate the accumulation of I element sense transcripts in the nuclei of the nurse cells; the asterisks (SF, $aub^{-/-}$) show the accumulation of functional I element transcripts at the anterior pole of the oocyte. (*C*) I strain ovary hybridized by two-color RNA FISH with I element (*red*) and Doc (*green*) antisense riboprobes. The yellow arrowheads indicate the colocalization of I element and Doc sense transcripts.

could be sequenced (2) in a strain (*Oregon R*) of unknown origin. We analyzed this stock and showed that it is R (data not shown), indicating that I element piRNAs can be present in R strains. We could not detect them in this strain by Northern blotting (data not shown). This suggests that I element piRNAs are indeed produced by the heterochromatic defective I elements in R strains but that their amounts are too low to allow detection by Northern blotting.

The piRNA Pathway Controls I Elements at the Posttranscriptional Level. To further investigate the repression mechanism of I elements by piRNAs, we first performed RT-PCR and quantitative RT-PCR experiments on total RNAs extracted from ovaries, using oligonucleotides specific of the euchromatic functional I elements (see *SI Text*). The results (Fig. S3) show that PCR products are detected in SF and *HT2* (I) but not in *JA* ovaries (see Fig. S3*A*). There is an approximately 40-fold increase of the signal in *HT2* ovaries compared to the background level provided by *JA* ovaries (see Fig. S3*B*). As expected, the increase is even higher (about 400-fold) in SF ovaries (data not shown). This indicates that I elements are transcribed in I females, although their steady-state transcript level is lower than in SF females.

The localization of these transcripts was determined by RNA FISH experiments on whole-mount ovaries using an I element antisense riboprobe. As a negative control, we used *JA* R females, devoid of functional euchromatic I elements, where no sense transcripts could be detected (Fig. 4*A*). In SF ovaries, the usual accumulation of such transcripts was observed in the oocyte (see Fig. 4*A*). Interestingly, numerous small dots were also lighted up in the nurse cell nuclei of SF females, which probably correspond to the nascent transcripts produced by the polytenized copies (21) of euchromatic functional I elements.

As usual, no transcript accumulated in the oocytes of I strain females (see Fig. 4*A*). However, some sense I element transcripts were detected in the nurse cells of these females, as judged by both the presence of the putative nascent transcripts and the presence of large intranuclear foci close to the nuclear membrane (see Fig. 4*A*). These foci were observed in more than 45% of the ovarioles of I females in six independent experiments. They did not colocalize with the nuclear Piwi protein (data not shown). These observations are consistent with the RT-PCR data (see Fig. S3), indicating that functional I elements are transcribed in I females, and they show that at least some of their transcripts are somehow stuck within the nuclei of the nurse cells.

A signal is also observed in the cytoplasm of the I nurse cells (see Fig. 4*A*) but not in the nuage at the outside periphery of the nucleus where most proteins involved in I element regulation are located. Functional full-length I element transcripts are not detected in I ovaries by Northern blotting (15), but when these functional transcripts are present (as in SF females) they are transported to the oocyte (17). The transcripts observed in the nurse cell cytoplasm of I females do not localize as functional transcripts (see Fig. 4*A*). We therefore suggest that they correspond to degraded I element RNAs.

piRNAs Are Involved in the Accumulation of I Element Transcripts within Nuclear Foci. To know if the accumulation of sense transcripts in the nucleus results from formation of double-stranded RNA, we performed two-color FISH on whole-mount ovaries with sense and antisense I riboprobes (Fig. S4). Interestingly, we observed I element antisense transcripts inside the nurse cell nuclei of all genotypes, including R females. However, even though they were found close to the nuclear membrane, like sense transcripts with which they sometimes overlap, they cannot be responsible for the accumulation of the sense transcripts in I ovaries because they hardly colocalized with the strongest sense RNA accumulation foci.

As shown above, the reduction of I element piRNAs accumulation in SF ovaries correlated with the export of the I sense transcripts from the nurse cell nuclei to the oocyte. To further investigate the possible interference of such piRNAs with the export of these transcripts, we tried to abolish the production of piRNAs in another way, different from hybrid dysgenesis. We first checked that, as expected from the derepression observed previously (see Fig. 1), the production of I element piRNAs dramatically decreased in *aub* mutant females (Fig. S5). The pattern of I sense transcripts in these ovaries turned out to be similar to that observed in SF dysgenic ovaries (see Fig. 4 *A* and *B*; Fig. S4): No accumulation of I element sense transcripts occurred in nurse cell nuclei whereas the putative nascent transcripts and the usual accumulation in the oocyte were observed (see Fig. 4*B* and Fig. S4). We conclude from these results that piRNA production results in accumulation of the transcripts of functional euchromatic I elements in the nurse cell nuclei.

To determine if the structures into which I element sense transcripts accumulate in nurse cell nuclei are sites of accumulation of repressed transposable elements, we studied the localization of the Doc retrotransposon RNAs (22). By two-color RNA FISH on whole-mount ovaries of I females, using I and Doc antisense riboprobes, we observed that I and Doc sense transcripts partially colocalize in common nuclear territories of the nurse cells (Fig. 4*C*).

Discussion

I Element piRNAs Are Associated with Nuclear Accumulation of Functional I Element Transcripts in the Female Germline. In the *Drosophila* ovary, piRNAs are an essential mediator of transposable element control (7, 9). To study the repression of multiple copies of euchromatic functional I elements in the germline of I strains, we took advantage of hybrid dysgenesis that allows to experimentally release I element expression and transposition in hybrid dysgenic females. We show here that this repression is related to the presence of piRNAs able to hybridize to I element transcripts. In the ovaries, the levels of repression of functional I elements correlate well with the amounts of I element antisense piRNAs: repression is total in I strain females that contain the highest level of piRNAs; it is abolished in SF females in which I element piRNAs are present in smaller amounts; and it is intermediate in the isogenic RSF females in which they are present at intermediate levels (see Fig. 2). Moreover, we show that this silencing is impaired in mutant ovaries for proteins involved in the piRNA pathway, in particular two proteins of the Piwi subfamily of the Argonaute family, Aub and Piwi, that are known to bind piRNAs (see Fig. 1). By contrast, mutations of genes involved in the siRNA- and miRNA-mediated silencing pathways do not affect I element repression.

Although piRNAs mediate chromatin modifications (10), we show that I element repression by the piRNA pathway occurs, at least partially, at the posttranscriptional level. Indeed, the sense transcripts of functional I elements are always detected in nurse cells, even in the repressive background of the I strains (see Fig. 4). The main differences between SF and I females are the amount and the fate of these transcripts: in SF females, they are exported outside the nurse cell nuclei and accumulate in the oocyte, whereas in I females they stay in the nurse cells. A fraction of the transcripts present in I female nurse cells accumulate in foci located close to the inner side of the nuclear envelope. This accumulation is correlated with the presence of antisense I element piRNAs in I ovaries and disappears when the amount of piRNAs is reduced by either hybrid dysgenesis or mutation of genes of the piRNA pathway, like *aub*. In such mutant ovaries, the sense transcripts are exported and transported toward the oocyte, just as in SF ovaries. Therefore, piRNAs look responsible for the nuclear accumulation of the sense transcripts synthesized by euchromatic functional I elements. The way they perform this role and the effector proteins are presently unknown.

Moreover, we have shown that transcripts of the Doc retrotransposon partially colocalize with those of the I element in foci of nurse cell nuclei (see Fig. 4*C*). This leads us to hypothesize that a general piRNA-mediated mechanism, resulting in accumulation of transposable element transcripts inside nurse cell nuclei, protects the genome against the deleterious effects of transposition in the germline.

Functional Euchromatic I Elements and a Maternal Component Are Required for Efficient Production of Antisense piRNAs. The mechanisms of piRNA biogenesis are not yet understood. It is assumed that piRNAs of each strand polarity result from the processing of longer transcripts by piRNAs of the opposite strand (2, 3) as illustrated by the ping-pong amplification model, which involves sense and antisense transcription of functional and defective transposable elements, respectively (see the introduction to this article). However, this model does not apply to the silencing of the *Drosophila* gypsy retroelement. Indeed, antisense gypsy piRNAs originate from gypsy-defective remnants located in the heterochromatic *flamenco* locus, but they are produced even in the absence of euchromatic functional gypsy elements (8, 9). By contrast, we show here that even though antisense I element transcripts are present in the ovaries of all strains including those of R females (see Fig. S4), R ovaries contain much lower levels of antisense piRNAs than the I ovaries (see Fig. 2). Our results clearly show that antisense piRNAs become detectable by Northern blotting only when functional I elements are added to a R genome, in an otherwise isogenic background (see Fig. 2). These data are in agreement with the ping-pong amplification model. The differences in the silencing of the gypsy and I elements might result from the fact that the control of gypsy occurs in the somatic follicle cells of the ovaries while the control of I is specific of the female germline, suggesting that the biogenesis of piRNAs differs according to ovarian cell lineages.

The amounts of I element piRNAs are strikingly different in the isogenic SF and RSF females (see Fig. 2), indicating that their production is not only determined by the presence of functional I elements but also depends on the maternal origin of the females. Maternal loading of small RNAs in early embryos has already been reported (23, 24). Maternal loading in embryos of small RNAs complementary to the *Penelope* retrotransposon was correlated with the repression of hybrid dysgenesis in *D. virilis* (25). We have checked that I element piRNAs are also present in early embryos in I strains (data not shown), and we report here that this kind of maternal effect can persist up to adulthood. Deposition of such RNAs might be responsible for the observed enhancement of piRNA accumulation in RSF females (issued from I females producing piRNAs) as compared with isogenic SF females (issued from R females producing no or much less piRNAs) (see Fig. 2). Similarly, the putative loading of small amounts of piRNAs in the oocytes of weak R strains might be able to trigger the observed accumulation in their SF

daughters of significant levels of piRNAs, mediating partial I element repression (see Fig. 3). Thus, the various levels of permissivity of R strains (reactivity), which are mostly maternally transmitted, would reflect the ability of R females to transmit piRNAs to their progeny. One should notice that both chromosomal and maternally transmitted components are also involved in the control in the female germline of the *Drosophila* P transposable element, responsible for another hybrid dysgenesis system, which appears to require genes of the piRNA pathway (26).

Materials and Methods

Detection of Small RNAs. The detection of small RNAs was done as described (9) with RNAs isolated from 100 pairs of ovaries. The I element antisense riboprobe was a 2.9-kb fragment corresponding to SP6 *in vitro* transcription of the pSPT19-I plasmid (15) digested by NheI. The sense riboprobe was obtained by T7 *in vitro* transcription of the same plasmid digested by PvuII.

RNA *in Situ* Hybridization. *In situ* hybridization experiments were performed as described (17) except for FISH, where the ovaries were not treated with proteinase K and where RNA instead of DNA probes were used. These probes were hydrolyzed 10 min at 60°C in 120-mM Na_2CO_3, 80-mM $NaHCO_3$. Hybridization was performed overnight at 65°C and washing at 55°C. No signal was observed after hybridization and washing at 42°C (data not shown), indicating that the signal observed after hybridization at 65°C reveals long, highly structured RNAs. The 2.9-kb I element antisense RNA probe described above was labeled using the Dig RNA labeling mix (Roche). The sense I element riboprobe was a 1.9-kb fragment labeled by T7 *in vitro* transcription of the PvuII- and ClaI-digested pSPT19-I plasmid, using a biotin RNA labeling mix (Roche). Two PCR products of the Doc element (coordinates 300-1300 and 1300–2300) were used as templates for T7 *in vitro* transcription with the biotin RNA labeling mix (Roche). The anti-digoxigenin antibody was labeled with rhodamine (Roche) (dilution 1/50) and the anti-biotin antibody with FITC (Vector laboratories) (dilution 1/100). Images were captured on a Zeiss LSM510 META confocal microscope. Image restoration was performed using the HRM interface running the Huygens 3.2 Software (Scientific Volume Imaging) and the Maximum Likelihood Estimation algorithm. Restored three-dimensional confocal stacks were then further analyzed using Imaris 6.1 (Bitplane) and the extended section tools.

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