

Argonaute protein PIWI controls mobilization of retrotransposons in the *Drosophila* male germline

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ABSTRACT

Proteins of the Argonaute family have been identified as key components of RNA interference (RNAi) pathway. RNAi-related mechanisms are implicated in the regulation of gene expression and repression of transposable elements in eukaryotes. The *piwi* gene encoding protein of the *Drosophila* Argonaute family was shown to be required for the germ stem cells maintenance. Here, we show that *piwi* is involved in silencing of LTR retrotransposons in testes. *piwi* mutations led to derepression of endogenous retrotransposon *copia* as well as to upregulation of the reporter gene driven by *copia* LTR. *piwi* mutation causes accumulation of retrotransposon *mdg1* transcripts at the apical tip of testes, including germinal proliferative center where PIWI protein was shown to be expressed. We applied inverse PCR approach to detect the newly arisen insertions of the *mdg1* retrotransposon in the progeny of individual *piwi* mutant males. Owing to *piwi* mutation a high rate of *mdg1* transpositions was revealed. Thus, *piwi* is involved in the silencing of retrotransposons in the precursors of male gametes. Our results provide the first evidence that protein of the Argonaute family prevents retrotranspositions. It is supposed that the disturbance of RNA silencing system in germinal cells might cause transposition burst.

INTRODUCTION

Gene silencing by homologous double-stranded RNA (dsRNA) is termed RNA interference (RNAi) (1). Long dsRNA is processed by Dicer enzymes into small interfering RNAs (siRNAs) that target repression of homologous sequences (2–4). siRNAs guide sequence-specific cleavage activity of the RNA-induced silencing complex (RISC) to

complementary mRNA (4–6) or induce chromatin-based events that result in transcriptional silencing (7–10). RNAi plays a role in defense against transposable elements and viruses, which can generate dsRNA (11–13). Antisense transcription and generation of hairpin structures in nascent transcripts are considered as a source of dsRNA of transposable elements in cells (14,15). Some RNAi-deficient mutants show a relief of transposon silencing in *Caenorhabditis elegans* (13,15–17). Mutations in genes that impaired RNAi cause an increase of retrotransposon transcript abundance in *Trypanosoma* (18), *Drosophila* (19–22) and mouse (23). Mobilization of retrotransposon was demonstrated in *Chlamydomonas* as a result of mutation in RNA-helicase encoding gene involved in the post-transcriptional silencing of transgenes (24). Silencing mediated by natural dsRNA has been implicated in the essential biological processes, including functioning of centromeres in *Schizosaccharomyces pombe* (8), development of macronucleus in *Tetrahymena* (25) and male spermatogenesis in *Drosophila melanogaster* (20,26,27). In these cases, dsRNA formation is provided by the transcription of the vestiges of transposable elements indicating that mechanisms of mobile elements silencing may be extended to the regulation of host genome.

Contribution of RNAi-based regulation to the retrotransposon-derived transcripts abundance in the *Drosophila* germline has been demonstrated (19–22), but no direct evidence in favor of the role of RNAi machinery in the control of their transposition rate has been obtained. Genetic control of retrotransposon mobilization in *Drosophila* is far from being understood. Spontaneous transposition rates of retroelements are usually low (28). However, a high level of retrotransposon mobility was detected in some stocks under particular genetic conditions. Mobility of *gypsy*, *ZAM* and *Idefix* retrotransposons was shown to be controlled by the yet uncharacterized X-linked *flamenco* locus (29,30). Non-LTR retrotransposon *I* is mobilized in the female germline as a result of definite outcrosses. This phenomenon is known as I–R hybrid dysgenesis (31). In this case, the mechanism of retrotransposon activation is also unclear. We revealed the effect of the RNAi genes *spn-E*, *aub* and *armi*

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on the *I* element expression in the female germline (22). We propose that hybrid dysgenesis associated with *I* element transposition burst may be caused by the impair of RNAi (22).

We investigated a role of the *piwi* gene in the control of retrotransposon mobilization in the male germline. *piwi* encodes a nucleoplasmic protein known to be required for self-renewal of stem cells in the male and female germline and the regulation of their division (32,33). Control of stem cell maintenance is the conserved developmental function of *piwi* and its orthologs in plants and mammals (34–38). PIWI is related to the highly conserved proteins of the Argonaute family. Argonautes are implicated in RNAi and related silencing pathways, including those that affect heterochromatin assembly and translational regulation via microRNA (miRNA)-directed pathway in different organisms [reviewed in (39–41)]. These proteins including PIWI are defined by two characteristic domains, PAZ and PIWI. PAZ domain recognizes the unique terminal structures of siRNAs (42,43) and, therefore, is essential for small RNA binding. The PIWI domain of Argonaute from the archaebacterium *Pyrococcus furiosus* is similar to a ribonuclease H domain (44), suggesting the role of Argonautes in the nucleolytic activity of RISC. However, only one of the four mammalian Argonaute 1 sub-family members (Ago2) was associated with the cleavage of target mRNA, suggesting that the other Argonautes might operate in different forms of RNA silencing (45). *Drosophila* Ago1 and Ago2 regulate the assembly of miRNAs and siRNAs, respectively, into RISC (46). *piwi* mutation relieves the transcriptional and post-transcriptional gene silencing of the multiple-copy transgenes (47) and impedes the repression of retrotransposon *gypsy* in the *D.melanogaster* genome (48).

In this study, we demonstrate that *piwi* mutations cause overexpression of retrotransposons *mdg1* and *cop1a* in the male germline, alteration of their expression pattern and mobilization of *mdg1* retrotransposon. Our results provide the first evidence that Argonaute family protein prevents mobilization of a retrotransposon. *cop1a* and *mdg1* are related to two major families of LTR retrotransposons, *Ty1/cop1a* and *Ty3/gypsy* groups, respectively. These groups are structurally differed by the location of integrase domain (49). The *Ty1/cop1a* group is termed the *Pseudoviridae*, reflecting structural similarity to retroviruses. Number of full-length copies of *cop1a* is 26 and *mdg1* is 13 according to the analysis of euchromatic part of the *Drosophila* genome (50). Spontaneous rate of transpositions is $\sim 10^{-4}$ per element per generation for *cop1a* (51), while no *mdg1* transpositions has been revealed in related stocks (28). It has been reported that conventional inbred crosses may generate spontaneous *cop1a* transpositions (52). We show that transposition burst of retrotransposon *mdg1* in the *Drosophila* germline is a result of mutation in the *piwi* gene involved in the stem cell development and RNA-mediated silencing phenomena. It is supposed that RNAi-related system of silencing controls transposition rate in the germline and its disturbance might cause transposition burst.

MATERIALS AND METHODS

Drosophila strains

Strain bearing *spindle-E* (*spn-E*) mutation was *ru¹ st¹ spn-E¹ e¹ ca¹/TM3, Sb¹ e^s*. *piwi* mutants were *piwi³* (PZ insertional

mutation) and *piwi²* (P-*ry11* transposon insertion) (32,53). *piwi²* mutation was balanced with the CyO, P{w⁺m = hsp70: GAL4} P{w⁺m = UAS: GFP}. *Su(var)2-5⁰¹*, *Su(var)3-9⁰³* and *E(z)⁶¹* alleles were used. P-element transformed flies carrying *cop1a*LTR-*lacZ* construct on the X and second chromosomes were kindly provided by E. G. Pasyukova (54).

In situ RNA analysis

In situ RNA analysis was carried out according to the procedure described previously (21). DIG-labeled riboprobes were synthesized according to the manufacturer's recommendations. PCR products carrying T7 promoter or plasmids containing the cloned fragments of retrotransposons were used as transcription templates. *mdg1*: 0.35 kb EcoRV–NsiI fragment from plasmid Dm688 (55) was subcloned into pBS SK⁻ and used to obtain sense or antisense riboprobe. *1731*: PCR-amplified fragment using primers (5'-ACGAATTCAGCGAACTGTCGTCG-3') and (5'-ATGGATCCATGTGACTGGTAGC-3') corresponding to 1138–1271 nt (GenBank accession no. X07656) was cloned into pTZ19R and used to detect sense *1731* transcripts. *cop1a*: 2.3 kb fragment from the 5' *cop1a* LTR to the EcoRI site into pBS SK⁻ was used to obtain a probe to detect sense or antisense *cop1a* RNA. *GATE*: PCR product carrying T7 promoter corresponding to 3055–3237 nt (GenBank accession no. AJ101298) was used for the detection of *GATE* sense RNA. PCR product carrying T7 promoter for *piwi* transcripts detection was amplified using primers 5'-GGCCGCGGACTTATGTGAGAGCAATGGATG-3' and 5'-GCCCGGCCACCGTCTCGATAAAATACG-3' corresponding to 7886–8519 nt (GenBank accession no. NG000285).

RT-PCR analysis

RT-PCR analysis was performed according to the protocol described previously (20). First strand of cDNA was synthesized using SuperScript II reverse transcriptase (Gibco BRL) and oligo(dT) primer according to the manufacturer's instructions. For PCR, the following primers were used: 5'-AAAC-TGGCCCCATTACCG-3' and 5'-CAAGTCCAGTTTCC-AGATG-3' corresponding to 180 752–180 967 nt fragment of constitutively expressed *Adh* gene (GenBank accession no. AE003410.1); 5'-GCATGAGAGGTTTGGCCATATAAGC-3' and 5'-GGCCCACAGACATCTGAGTGTACTACA-3' corresponding to 1685–1903 nt for *cop1a* (GenBank accession no. M11240); 5'-CCTAAGGAATTAGGGTGGTCCTAAGT-TTAC-3' and 5'-TTCAAAGGAGGGAGATGTAGTATAT-ACG-3' corresponding to 7023–7166 nt for *mdg1* (GenBank accession no. X59545). Results of RT-PCR analysis was evaluated using ImageQuant5.2.

X-gal staining

X-gal staining of testes was performed according to the protocol described previously (20).

Inverse PCR and detection of the *mdg1* transpositions

DNA samples isolated from the progeny of individual crosses of female *piwi³/+* to *piwi³/piwi³* or *piwi³/+* males were analyzed. DNA was isolated according to the standard method (56) from individual F1 and parental flies after gonad removal.

DNA (equivalent to one-fourth of a fly, ~0.2 µg) was digested by HhaI restriction enzyme recognizing 4-bases site in the 5'-untranslated region (5'-UTR) of *mdg1*. After inactivation of enzyme, restriction fragments were ligated overnight in a 100 µl in the presence of 1 U of T4 ligase (Promega) and precipitated. DNA (1/30 of fly per reaction) was PCR amplified using *mdg1*-specific primers: 5'-CGA-TCTGAGTGAGTAGAGTGTGAGTGTG-3' and 5'-CCTA-AGGAATTAGGGTGGTCTAAGTTTAC-3' [according to *mdg1* (GenBank accession no. X59545)]. At the annealing temperature of 60°C, 35 cycles were performed in the presence of 5 µCi [α -³²P]dATP per reaction. Samples were loaded on a 5% denaturing acrylamide gel and products were visualized by using autoradiography. To detect the sites of the *mdg1* transpositions, the new bands, pointing to new insertions, were cut from the gel, eluted and amplified using primers 5'-ATGGATCCGTGTGAGTGTGATGGGAAAAACAG-3' and 5'-ATGAATTCGGGTGGTCTAAGTTTACTTATTA-GTTGAC-3' [according to *mdg1* (GenBank accession no. X59545)]. Cloning and sequencing of three products allowed us to identify the region of insertions on the X, second and third chromosomes (GenBank accession nos AC104515, AC007291 and AC010011, respectively). Three primers corresponding to the region of insertions (5'-AAGCACAATTCCTCTCCATAACTTTG-3'; 5'-ACGCGTTGAAGTTT-AAGTCAACATG-3'; 5'-GCACGAAATGGTATCATCAG-TTTATC-3') and primer 5'-CCTAAGGAATTAGGGTGGT-CCTAAGTTTAC-3' corresponding to *mdg1* LTR were used to demonstrate the presence of insertions in F1 DNA samples and their absence in parental DNAs.

RESULTS

The *piwi* gene is indispensable for silencing of *mdg1* and *copia* LTR retrotransposons in testes

We investigated the effect of *piwi* mutations on the expression of LTR retrotransposons in testes by *in situ* RNA analysis using strand-specific riboprobe. *piwi*² and *piwi*³ mutations are caused by the P-element insertions into the coding and 5'-UTRs of the *piwi* gene, respectively (32). The PIWI protein expression is known to be attributed to a germinal proliferative center at the apical tip of larval and adult testes containing somatic hub cells and mitotically dividing germ stem cells (32,33) (see Figure 1A). *piwi* transcripts were revealed by *in situ* RNA hybridization using *piwi* riboprobe in the region of germinal proliferative center of the *piwi*^{3/+} adult and *piwi*^{2/+} larval testes (Figure 1B, arrows) in accordance with the observations reported previously (33). The absence of *piwi* transcripts in the larval *piwi*^{2/piwi}² and adult *piwi*^{3/piwi}³ testes is closely correlated with the emergence of the *mdg1* sense transcripts at the tip regions of testes (Figure 1B, arrows). At the same time, in the remaining part of testes, the level and pattern of the sense *mdg1* transcripts is roughly similar in the *piwi*^{3/+} and *piwi*^{3/piwi}³ males (Figure 1B and C). The *mdg1* transcripts are localized in spermatocyte nuclei, near the DAPI stained compact regions of chromatin (Figure 1B, inset). Sense *copia* transcripts are detected in a limited number of spermatocytes in testes of *piwi*^{3/+} males (Figure 1B). *copia* sense transcript accumulation in testes of homozygous *piwi*³ males is detected in the basal part of testis where postmeiotic

stages of spermatogenesis are known to have occurred (Figure 1B). No *copia* sense transcripts are detected at the apical tip of testes. A significant increase in *copia* transcript abundance was also observed in spermatocytes of larval testes of homozygous *piwi*² males as compared with heterozygous *piwi*^{2/+} males (Figure 1B). Thus, the derepression of *copia* in *piwi* mutants was observed at the later stages of spermatogenesis, where PIWI protein has not been detected (33). *mdg1* and *copia* antisense transcripts are not detected by *in situ* RNA hybridization (Figure 1B). RT-PCR analysis confirmed the derepression of *copia* in testes of *piwi* mutants (Figure 1C). Amount of *copia* and *mdg1* transcripts, normalized against *adh* transcripts, increases in testes of *piwi*^{2/piwi}² males by 1.9 and 1.3 times, respectively. It is not surprising that the local accumulation of *mdg1* transcripts in the region of stem cells exerts no noticeable effect on their total amount evaluated by RT-PCR (Figure 1C).

LTR retrotransposons *1731* and *GATE* have been shown to be transcribed in primary spermatocytes (21,57), but the level of transcripts of these elements detected by *in situ* RNA hybridization was not affected by *piwi* mutation (data not shown).

It has been reported that mutations of some *Drosophila* RNAi genes and *piwi* cause mislocalization of heterochromatic proteins HP1, HP2 and a loss of histone H3 methylation in nuclei of somatic cells (58), suggesting the role of RNAi machinery in heterochromatin assembly and transcriptional gene silencing. Thus, PIWI might be proposed to be a component of chromatin silencing complex that induces transcriptional repression of transposable elements. However, no effects of mutations in genes encoding HP1 [SU(VAR)02-05] and histone H3 methyltransferases SU(VAR)3-9 and E(Z) on the expression of *copia* and *mdg1* were revealed in testes by *in situ* RNA analysis (data not shown).

piwi mutation leads to the derepression of a *copia* LTR-*lacZ* fusion construct in testes

To study whether *copia* LTR is a target of silencing mediated by *piwi*, we used a construct containing full-size *copia* LTR fused to *lacZ* (*copia*LTR-*lacZ*). *copia* LTR comprises the known upstream regulatory region, including 137 bp of the transcribed fragment (54) (Figure 2A). We introduced the X-chromosome containing *copia*LTR-*lacZ* into the stock carrying *piwi*² mutation. The level of reporter gene expression is significantly increased in *piwi*^{2/piwi}² testes. No staining at the apical tip of testes is revealed (Figure 2B, arrows). *lacZ* expression is detected in early spermatocytes as well as in the basal parts of *piwi*² homozygous testes where the accumulation of endogenous *copia* transcripts was observed (Figures 1B and 2B). The difference in the expression patterns of endogenous *copia* and *copia*LTR-*lacZ* might be related to the absence of some *copia* regulatory regions in the transgene as compared with full-size endogenous copies.

To obtain additional arguments in favor of a relief of *copia*LTR-*lacZ* expression owing to disturbance of RNA silencing, we tested the effect of mutation of RNAi gene *spn-E*. *spn-E* encodes putative RNA-helicase, and was shown to be responsible for the production/stabilization of siRNA necessary to silence the *Stellate* repeats in testes (27). *spn-E* mutation causes accumulation of *copia* transcripts

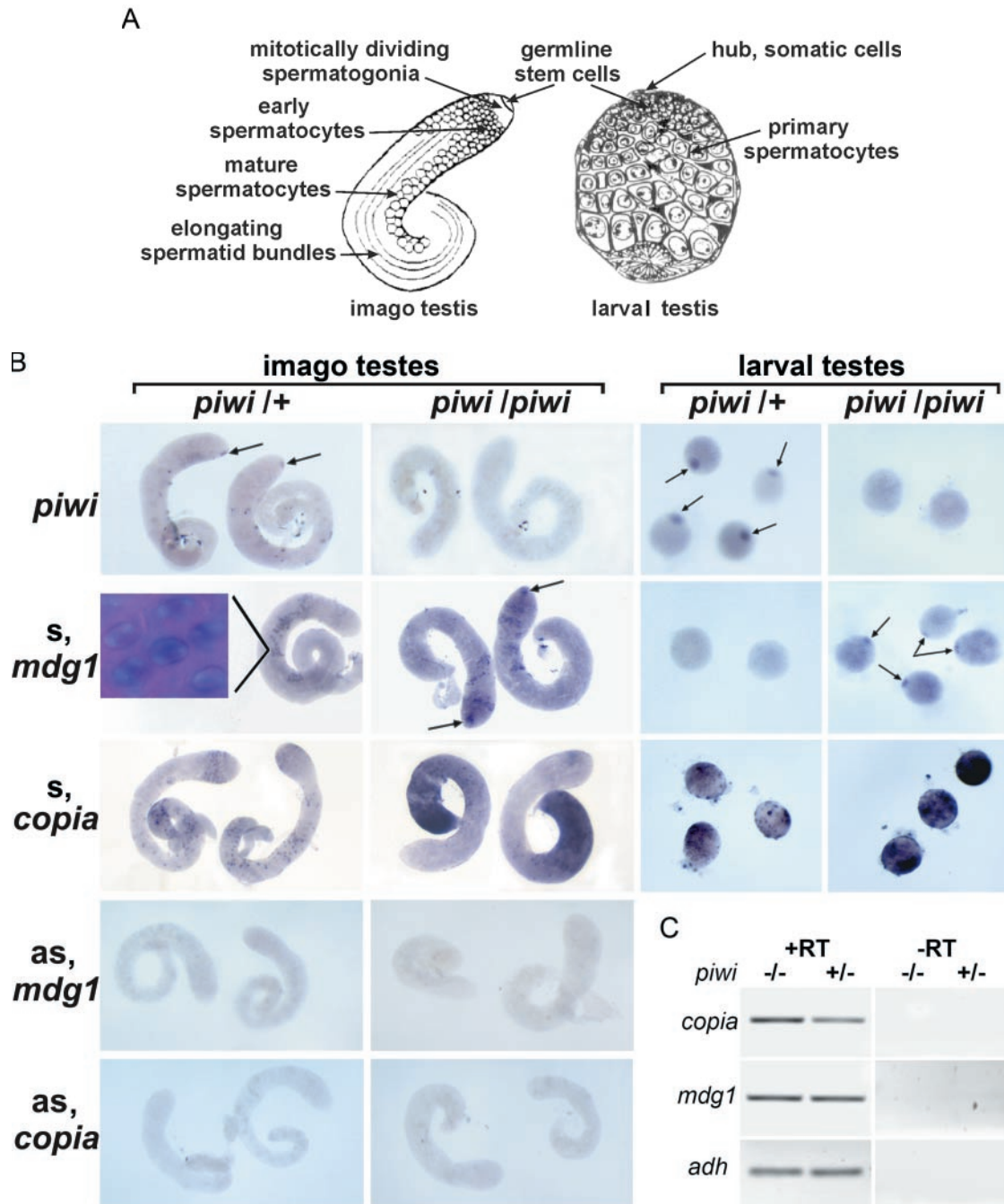


Figure 1. *piwi* mutation relieves silencing of retrotransposons *mdg1* and *copia* in the testes. (A) Diagrammatic representation of imago and larval testes (out of scale) [according to (66) and (67), respectively]. The apical tip of adult testis contains mitotically dividing stem cells and spermatogonial cells; spermatocytes and elongating spermatid bundles are indicated. Tip of larval testis contains the somatic hub cells and the germline cells where PIWI protein is expressed (33). Larger cells of larval testes are represented by mature spermatocytes. (B) *In situ* RNA hybridization to *piwi*^{3/+} and *piwi*^{3/piwi}³ adult and *piwi*^{2/+} and *piwi*^{2/piwi}² larval testes of riboprobes detecting sense (s) and antisense (as) transcripts. Genotypes are indicated on the top of the panels; probes are indicated on the left. Arrows show *piwi* and *mdg1* transcripts location in proliferative center. Absence of *piwi* transcripts in *piwi*^{3/piwi}³ adult and *piwi*^{2/piwi}² larval testes is correlated with a relief of the *mdg1* expression in the homozygous *piwi* mutants. Inset: *mdg1* sense transcripts are detected in spermatocyte nuclei. DAPI staining visualizes compact regions of chromosomes in maturing spermatocytes; *mdg1* transcripts are detected in non-DAPI stained region of spermatocyte nuclei. *copia* transcripts are not detected at apical tip. *copia* expression is enhanced in spermatocytes of *piwi*^{2/piwi}² larval testes and in mature spermatocytes and postmeiotic cells of the adult *piwi*^{3/piwi}³ testes. No antisense (as) *mdg1* and *copia* transcripts are revealed in *piwi*^{3/+} and *piwi*^{3/piwi}³ adult testes. (C) RT-PCR analysis of *copia* and *mdg1* expression in adult testes of *piwi*^{3/+} and *piwi*^{3/piwi}³ males. Coamplification with alcohol dehydrogenase (*adh*) primers was used to control RNA quantity.

in testes (19). Expression of the *copia*LTR-*lacZ* reporter constructs located on the X or second chromosomes is significantly increased in testes of the homozygous *spn-E*¹ males (Figure 2C). The patterns of the *lacZ* expression in testes of the

*spn-E*¹ homozygous males are similar to the observed distribution of *lacZ* expression in testes of *piwi*² homozygous males. Thus, *copia* LTR contains regulatory sequences that confer *spn-E* and *piwi* dependence of retrotransposon repression.

***mdg1* transposes in the *piwi*³ male germline**

The *mdg1* overexpression in *piwi* mutants is observed in the germinal stem cells, suggesting PIWI protein involvement in the repression of transpositions in the precursors of male

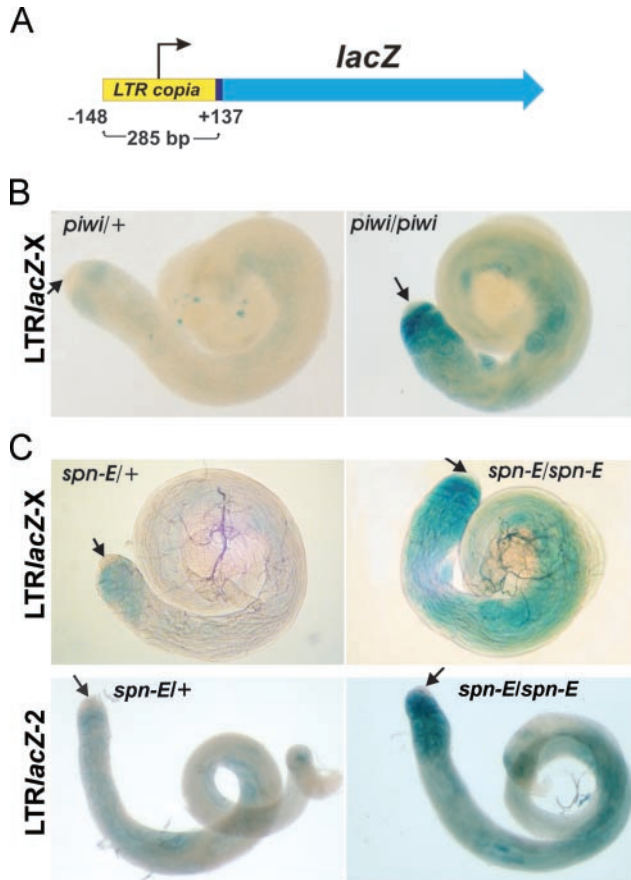


Figure 2. Overexpression of the *copia*LTR-*lacZ* construct in *piwi*² and *spn-E*¹ testes. (A) The *copia*LTR-*lacZ* construct comprises full-size *copia* LTR fused to the *lacZ* reporter. (B) X-gal staining of testes from *piwi*² males carrying the *copia*LTR-*lacZ* construct on the X-chromosome. (C) X-gal staining of testes from *spn-E*¹ males carrying the *copia*LTR-*lacZ* construct on the X (upper panels) and second chromosomes (lower panels). A level of *lacZ* expression is greatly increased in the homozygous *piwi*² and *spn-E*¹/*spn-E* testes as compared with heterozygous *piwi*² and *spn-E*¹/. Arrows indicate the apical tips of testes, where β -galactosidase activity is not detected.

gametes. To check the *mdg1* transpositions, single *piwi*³ homozygous males were crossed to the individual *piwi*³/*+* females. A comparative inverse PCR analysis was applied to the single F1 progeny flies and their parents. Inverse PCR approach allows us to estimate the copy number of transposable elements and to detect the newly arisen insertions. Figure 3 shows the rationale of the method. The number of full-length and partial euchromatic *mdg1* copies per *D.melanogaster* genome amounts to 25 copies according to the Release 3 of euchromatic genome sequence (50). We detected ~35 PCR bands per genome and this surplus number of copies may be contributed by the heterochromatic part of the genome. Our estimation of the number of *mdg1* copies corresponds well with the results obtained by using Southern-blot analysis (59). Differences between the patterns of PCR bands detected in individual parental DNAs reflect the polymorphisms of *mdg1* sites peculiar to the *piwi* and balancer chromosomes as well as to the presence of the Y-specific bands in male DNA (Figure 4A and B). The new bands in F1 DNA samples that are non-detected in parental DNAs demonstrate the occurrence of transposition events (Figure 4A). We failed to reveal newly appeared PCR bands in the progeny of *piwi*³/*+* males (Figure 4B), while the number of new insertions in the progeny of the *piwi*³/*piwi*³ males amounts to 19 per 101 flies analyzed (Table 1). To confirm the emergence of the new bands as the result of transpositions, three new bands detected in three individuals were cloned and sequenced. The results of sequencing allowed us to attribute the *mdg1* insertions in three F1 individuals to the regions of unique genomic DNA on the X, second and third chromosomes. PCR analysis of DNA from F1 and parental flies using primers specific to the insertion sites and the *mdg1* LTR demonstrates the occurrence of insertions in F1 samples and 'empty sites' in the parental DNAs (Figure 4C). The average frequency of *mdg1* transposition in testes of *piwi*³ mutants amounts to 0.19 transpositions per generation. However, this value may be underestimated as a result of low resolution of high-molecular weight PCR bands. The new PCR bands of the same size were observed in F1 individuals. At least some of these bands may correspond to identical *mdg1* insertions, indicating the presence of the germ cell clusters carrying identical sites of insertions and suggesting that transpositions occur at the premeiotic stages of spermatogenesis.

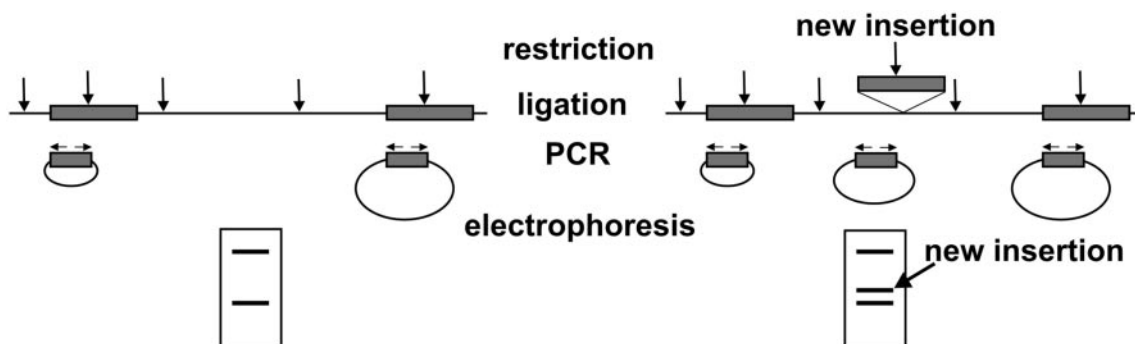


Figure 3. Detection of the retrotransposon insertions by inverse PCR analysis. Gray boxes represent transposable elements; restriction sites are indicated by vertical arrows and horizontal arrows designate primers. Restriction-ligation of DNA samples followed by PCR using retrotransposon-specific primers revealed PCR products corresponding to insertions of retrotransposon.

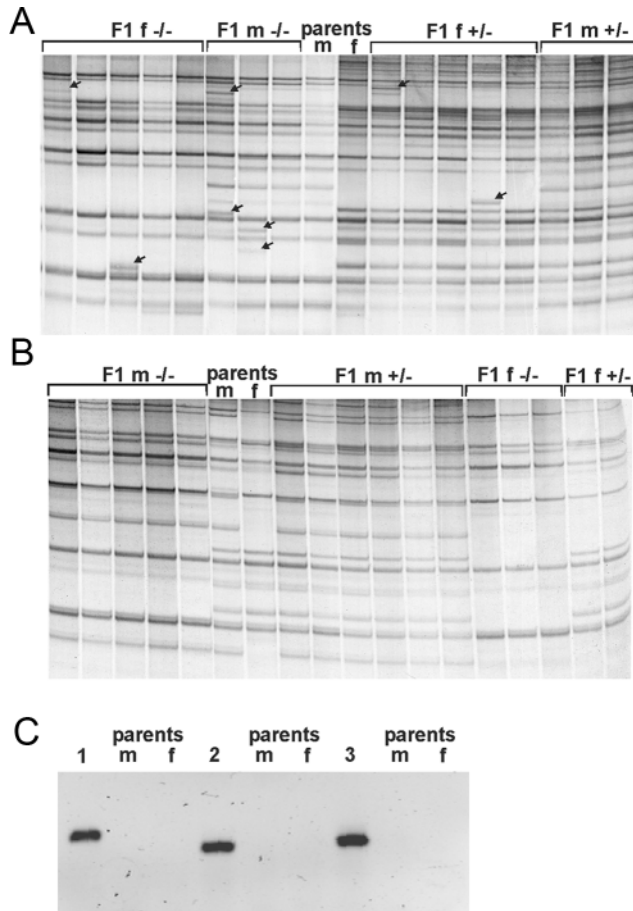


Figure 4. Mobilization of retrotransposon *mdg1* in testes of *piwi* mutants. Genomic DNA from individual flies was digested, ligated and PCR amplified using *mdg1*-specific primers. Genomic distribution of *mdg1* copies in DNAs from individual F1 flies are shown to the left and to the right. Pattern of PCR bands of parents are in the middle (m, males; f, females; $-/-$, *piwi³/piwi³*; and $+/-$, *piwi³/+*). (A) Parents are *piwi³/piwi³* males and *piwi³/+* females. Mobilization of *mdg1* is demonstrated by the new bands revealed in progenitor DNAs (indicated by arrows), which are not detected in parental DNAs. (B) Parents are *piwi³/+* males and *piwi³/+* females. No new bands differing from parental DNAs were detected in F1 DNA. (C) Detection of *mdg1* insertion sites. PCR analysis of DNA from F1 (1, 2 and 3) and parental flies (m and f) using specific primers corresponding to the insertion sites in the X, second and third chromosomes, respectively, and the *mdg1* LTR primer. Appearance of insertions in F1 samples (bands) and 'empty sites' in parental DNAs are shown.

Table 1. Estimation of the *mdg1* mobility in testes of *piwi³/piwi³* ($-/-$) and *piwi³/+* ($+/-$) males

Male genotype	Number of analyzed F1 individuals	Number of new PCR bands
$-/-$	50	9
$-/-$	51	10
$+/-$	35	0
$+/-$	16	0

DISCUSSION

We presented evidence that the *piwi* gene, coding a member of the Argonaute protein family, is required for the silencing of the retrotransposons in the *Drosophila* male germline. *piwi*

mutation causes the derepression of retrotransposon *mdg1* in the germinal proliferative center and, as a result, relieves transpositions of this mobile element. This observation is the first evidence that mutation in a single gene triggers retrotransposition in the *Drosophila* germline. We suggest that activation of retrotransposon caused by *piwi* mutation occurs as a result of the RNAi machinery disturbance. Strong evidence in favor of *Drosophila* PIWI as a component of RNAi is not obtained, but it has been reported that *piwi* mutation causes a relief of transgene repeats silencing and diminishing transgene-related siRNA (47). We failed to detect *copia* and *mdg1* antisense transcripts by *in situ* RNA hybridization suggesting the low level of these transcripts in testes. Antisense transcripts corresponding to LTR sequence of *copia* were revealed in ovaries (M. S. Klenov, unpublished data). siRNAs corresponding to transposable elements including *copia* have been detected in *Drosophila* testes (60) supporting the participation of RNAi-related mechanism in retrotransposon silencing.

Here, we demonstrated that *lacZ* expression driven by the *copia* LTR is upregulated in testes of RNAi mutants. *piwi* and *spn-E* mutations have similar effect on a reporter gene expression, suggesting the participation of both genes in the same silencing pathway. Thus, LTR is considered as a target of silencing machinery. The role of LTRs in RNAi-mediated regulation of retrotransposons and adjacent host genes in yeast has been emphasized (9). The most abundant repeat-associated siRNAs were related to the LTR of multicopy retrotransposon *roo* in *D.melanogaster* (60).

Presence of PIWI protein in embryos is restricted to the presumptive gonad. In adults, PIWI is required in somatic signaling cells to maintain germline stem cells (33). Our results show the role of *piwi* in silencing of the *mdg1* retrotransposon in the germinal proliferative cells and *copia* at the late spermatocytes and postmeiotic stages where PIWI protein is not detected. Thus, PIWI presumably affects silencing mechanisms that are operated at the later stages of spermatogenesis. Effect of *piwi* may be indirect and mediated through the other targeted genes operating on chromatin level. Actually, the role of PIWI, in concert with HP1 and histone H3 methylation, in the epigenetically inherited silencing has been reported previously (58). However, we revealed no effect of HP1 and SU(VAR)3-9 and E(Z) histone H3 methylases mutations on the expression of *mdg1* and *copia* retrotransposons in testes. Further experiments will elucidate the mechanism of PIWI-mediated retrotransposon silencing.

Transcript abundance of retrotransposons *1731* and *GATE*, which are active in primary spermatocytes (21,57) is not affected by *piwi* mutation. It is significant that retrotransposon transcript abundance may not correlate with retrotransposition frequency that is dependent on the DNA copy production and immediate insertion. It has been shown that mutations of RNAi genes in *C.elegans*, including putative RNA-helicase gene, have resulted only in the activation of the selected DNA transposons (15,61). The similar effects of RNAi silencing affecting selective mobile elements in plant genome have been also reported previously (62). All these results argue against universal RNAi-dependent silencing of retrotransposons. Specific pattern of dsRNA formation, which triggers the RNAi pathway, might be responsible for these differences. Moreover, distinct members of Argonaute family may act in

distinct silencing pathways at different developmental stages. Ago2 is essential for siRNA-directed RNA cleavage in *Drosophila* embryos (46). The regulatory effect of the *piwi* gene may be realized upstream of the *ago2* in the respective silencing mechanism in the germline. Alternatively, both *piwi* and *ago2* products may be indispensable to organize specific RISC complex directed to retrotransposon silencing. In this case, *piwi* mutation may compromise the functioning of this complex.

We revealed the effect of *piwi* mutation on the *mdg1* mobilization and the derepression of *copia* in the male germline. Involvement of the other RNAi genes (*spn-E*, *aub* and *armi*) have been revealed in the control of expression of different families of transposable elements in the *Drosophila* female germline, including the non-LTR retrotransposons *HeT-A* and *I* (22). At the same time, a growing body of evidence demonstrates a profound contribution of the RNAi mechanism to the regulation of host development. RNAi genes, including *spn-E*, *aub* and *armi*, were shown to be responsible for specifying the axes of developing oocyte and embryonic patterning in *Drosophila* (63–65). RNAi genes are proposed to prevent premature translation of the key embryonic determinant *oscar* via miRNA pathway (65). Thus, a dual function of RNAi-related genes implicated both in the developmental control and in the retrotransposon silencing is unveiled. We suggest that the same RNAi-related mechanism mediated by naturally occurring dsRNA via PIWI protein assistance suppresses mobile element transpositions and regulates host genes ensuring stem cell renewal in testes. Further refining studies are needed to reveal the mechanism ensuring this dual function of RNAi protein complexes.

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