Mechanism of the piRNA-mediated silencing of *Drosophila* telomeric retrotransposons

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ABSTRACT

In the *Drosophila* germline, retrotransposons are silenced by the PIWI-interacting RNA (piRNA) pathway. Telomeric retroelements *HeT-A, TART* and *TAHRE*, which are involved in telomere maintenance in *Drosophila*, are also the targets of piRNA-mediated silencing. We have demonstrated that expression of reporter genes driven by the *HeT-A* promoter is under the control of the piRNA silencing pathway independent of the transgene location. In order to test directly whether piRNAs affect the transcriptional state of retrotransposons we performed a nuclear run-on (NRO) assay and revealed increased density of the active RNA polymerase complexes at the sequences of endogenous *HeT-A* and *TART* telomeric retroelements as well as *HeT-A*-containing constructs in the ovaries of *spn-E* mutants and in flies with *piwi* knockdown. This strongly correlates with enrichment of two histone H3 modifications (dimethylation of lysine 79 and dimethylation of lysine 4), which mark transcriptionally active chromatin, on the same sequences in the piRNA pathway mutants. *spn-E* mutation and *piwi* knockdown results in transcriptional activation of some other non-telomeric retrotransposons in the ovaries, such as *I*-element and *HMS Beagle*. Therefore piRNA-mediated transcriptional mode of silencing is involved in the control of retrotransposon expression in the *Drosophila* germline.

INTRODUCTION

Silencing of mobile elements in germ cells depends on a distinct class of 24- to 30-nt long RNAs called PIWI-interacting RNAs (piRNAs) associated with Argonaute proteins from the PIWI subfamily (1,2). piRNA pathway mutations lead to overexpression and mobilization of retrotransposons in the germline (1,3,4). However, the mechanism of the piRNA-mediated gene silencing in *Drosophila* remains obscure. Short RNAs were shown to target the associated protein complex to degrade complementary mRNA (5). Short-RNA-mediated heterochromatin assembly was described in fission yeast, plants, and ciliates (6–8). In this case, short RNAs guide histone methyltransferase to the target locus to methylate lysine 9 of histone H3 (H3K9) with subsequent binding of heterochromatic protein 1 (HP1) or its homolog (Swi6 in fission yeast). Heterochromatization diminishes the transcriptional capacity of the target locus resulting in transcriptional silencing. How do piRNAs maintain transposon silencing in the germline? In mice, transposon-specific piRNAs drive transposon promoter methylation in the male germline (2,9). The three *Drosophila* PIWI proteins, PIWI, Aubergine (Aub) and Argonaute3 (Ago3) which are found in the germline, are associated with short RNAs *in vivo* and demonstrate cleavage activity of the complementary RNA *in vitro*, implicating their involvement in the post-transcriptional degradation of mRNA (1,10,11). Despite numerous studies describing the piRNA-mediated repression of retrotransposons in the *Drosophila* germline, only a few of them suggest mechanism of repression. piRNA-mediated post-transcriptional destabilization of retroelement transcripts was shown (12). A post-transcriptional mechanism was proposed to be involved in the piRNA-mediated silencing of testis-expressed *Stellate* repeats (13) and the non-LTR retrotransposon *I*-element (14). At the same time, the piRNA-mediated silencing by transgenes located at subtelomeric regions was shown to be dependent on such heterochromatic components as HP1 or its partner, Su(var)3–7 (15). Changes of the chromatin structure of retroelements caused by piRNA pathway mutations in *spn-E* gene (16) also indicate that piRNAs may target chromatin modifications. Most probably, both

transcriptional and post-transcriptional pathways may be involved in the piRNA-mediated silencing of the same target gene. Klattenhoff and Theurkauf (4) proposed a model of compartmentalization of the piRNA pathway. According to this model, cytoplasmic proteins Aub and Ago3 catalyze the RNA cleavage in the cytoplasm, most likely in the perinuclear structure nuage operating at the post-transcriptional level. Being localized in the nucleus, PIWI might be involved in the transcriptional silencing of its target locus. Based on the well-known scheme of the siRNA-mediated heterochromatin formation in yeast, there were attempts to directly connect piRNA pathway components with heterochromatin marks such as HP1 and histone H3 modifications (H3K9Me2 and H3K9Me3) in *Drosophila*. Such approach results in the appearance of several contradicting facts. PIWI was shown to be a component of chromatin (17) which mediates HP1 recruitment to the heterochromatic loci (18,19). On the other hand, PIWI was described as a nucleoplasmic protein which is not associated with chromatin (16,20). Formation of heterochromatin and its association with HP1 was shown to be independent of the piRNA pathway (21). A reasonable explanation for this apparent contradiction is that different short RNA targets are regulated by distinct mechanisms depending of their genomic location. In our study we decided to directly estimate the transcriptional status of retroelements in the germline of the piRNA pathway mutants rather than to look for the vestiges of known chromatin modifying pathways in attempt to connect them with piRNA pathway. The main objects of this study are the *Drosophila* telomeric retrotransposons.

Telomeres in *Drosophila* are maintained by transpositions of specialized telomeric retroelements rather than by the telomerase activity that adds short DNA repeats to chromosome ends in other eukaryotes. *HeT-A*, *TART* and *TAHRE* non-LTR retrotransposons are found at *Drosophila* telomeres in mixed tandem head-to-tail arrays; their 3′-ends are orientated towards the centromere (22–24). *HeT-A* and *TART* elements were shown to be transcribed bidirectionally from internal sense and antisense promoters (25–27). The abundance of telomeric retroelement transcripts, both sense and antisense, as well as the frequency of their transpositions on the chromosome ends, is controlled by a piRNA-mediated mechanism (28,29). The mechanism of piRNA-mediated telomeric retrotransposon silencing in the *Drosophila* germline remains elusive. The question about the mechanism of the telomeric retroelement silencing is of great interest because, in the case of transcriptional silencing, it might concern such an important process as formation of the telomeric chromatin which protects chromosome ends from fusion and is involved in meiotic and mitotic telomere behavior in different organisms. In *Schizosaccharomyces pombe*, centromeric and subtelomeric repeats of the transposon origin impact the siRNA-mediated spreading of heterochromatin through the activity of HP1 family proteins, histone methyltransferases, and the histone deacetylase complex (30–32).

Here, we have demonstrated that expression of the reporter genes driven by the *HeT-A* promoter is activated in piRNA mutants independently of where a reporter is located, in euchromatin or at the chromosome end. Furthermore, nuclear run-on analysis (NRO) was applied to determine directly whether the piRNAs induce transcriptional silencing of the telomeric retrotransposons. We have demonstrated piRNA-mediated transcriptional repression of endogenous *HeT-A* and *TART* retroelements, *HeT-A*-containing constructs located out of natural telomeres as well as some other non-telomeric retrotransposons. The data of NRO assay are in accord with chromatin immunoprecipitation (ChIP) analysis of histone H3 modifications which are characteristics of transcriptionally active loci. Although the protein machinery that transmits a signal from piRNA to chromatin complexes remains elusive, these data provide strong evidence of the piRNA-mediated transcriptional silencing in the *Drosophila* germline.

MATERIALS AND METHODS

Drosophila strains

Strains bearing *spindle-E* (*spn-E*) mutations were ru^1 st^1 $spn-E^1$ e^1 ca^1/TM3, Sb^1 e^s and ru^1 st^1 $spn-E^{hls3987}$ e^1 ca^1/TM3, Sb^1 e^s. *aub* mutants were aub^{QC42}/CyO and aub^{HN}/CyO. *vasa* locus mutations were vig^{EP812} and vas^{PH165}. These mutations affect two genes, *vig* and *vasa*, located at the same locus (33,34). We used $piwi^2$ and $piwi^3$ mutations. P-element-mediated germ line transformation of Df $(1)w^{67c23(2)}$, *y* embryos was performed according to standard protocol (35). Multiple transgenic *Drosophila* lines were established (Supplementary 'Materials and Methods' section). *si_piwi* flies were F1 of the crossing between #101658 line bearing *piwi* hairpin construct (VDRC Stock Center) and #25751 line (P{UAS-Dcr-2.D}1, w^{1118}, P{GAL4-nos.NGT}40, Bloomington Stock Center) providing GAL4 expression under the control of the germline-specific promoter of *nanos* gene. Control flies were F1 of the crossing of #60000 (w^{1118}, VDRC Stock Center) and #25751 (Bloomington Stock Center).

Reporter construct design

Constructs were made in the CaSpeR-AUG-β-gal vector. The *HeT-A* sequence was derived from clone z2 containing the fragment of the *HeT-A* element attached to a terminal deleted X chromosome (29). Clone z2 was used as a template to amplify 434 bp of the *HeT-A* promoter region. *HeT-A* PCR fragment obtained with 5′-ATGAAT TCATCCATCGCCCGCAACATG-3′ and 5′-ATGGAT CCTTTGCTGGTGGAGGTACGGAGAC-3′ primers was inserted into the EcoRI and BamHI polylinker sites of CaSpeR-AUG-β-gal.

In situ RNA hybridization, DNA FISH and immunohistochemistry

In situ RNA analysis was carried out according to the previously described procedure (36) using DIG-labeled strand-specific *HeT-A* riboprobes and alkaline phosphatase (AP)-conjugated (diluted at 1/2000) anti-digoxigenin (DIG) antibodies (Roche). The *HeT-A* probe contained a

fragment of the ORF (nucleotides 4330–4690 of the GenBank sequence DMU06920). The combination of protein and DNA localization was done according to the previously described procedure (37). Primary polyclonal rabbit anti-HP1 antibodies (Covance, diluted at 1:1000) were used. The probes used for DNA FISH analysis were: *yellow*, PCR amplified fragment using primers 5′-CTGATCACCTTGGTGACGCCG-3′ and 5′-AATTCGCGTATCCGTGGTCAAGTC-3′; *TART*, cloned fragment of *TART* ORF2, corresponding to 434–2683 nt in GenBank sequence DMU02279; *HeT-A*, cloned fragment of *HeT-A* ORF, corresponding to 1746–4421 nt in GenBank sequence DMU 06920. To stain DNA, ovaries were incubated in PBS containing 0.5 μg/ml DAPI. The confocal microscope, Zeiss LSM 510 Meta, was used to visualize the ovaries.

NRO assay

Ovaries from ~300 females were dissected and homogenized with a Dounce homogenizer (Sigma) in 300 μl of buffer HB [15 mM HEPES, pH 7.5, 10 mM KCl, 2.5 mM MgCl$_2$, 0.1 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.05% NP40, 1× complete protease inhibitor (Roche)] containing 0.35 M sucrose. All steps of nuclei isolation and washing were done on ice with ice-cold solutions. Homogenized solution was filtered through Miracloth membrane (Calbiochem). The filtrate was overlaid on a cushion containing 0.8 ml of 0.8 M sucrose-HB as the bottom phase and 0.3 ml of 0.35 M sucrose-HB as upper phase and centrifuged at 10 000 rpm for 10 min in Eppendorf centrifuge at 4°C. The supernatant was discarded and nuclei pellet was resuspended in 0.5 ml of RB buffer (5 mM Tris, pH 8.0, 5 mM MgCl$_2$, 150 mM KCl, 1 mM DTT, 1× complete protease inhibitor (Roche) and centrifuged at 6000 rpm for 10 min at 4°C. The pellet was resuspended in 100 μl RB containing 0.5 mM of each rATP, rGTP, rCTP, 5-bromouridine 5′-triphosphate (BrUTP, Sigma) and 1 U/μl of RNasin (Promega) and was incubated at 25°C for 30 min. After the run-on reaction RNA was isolated using TRIzol LS reagent (Invitrogen) according to the manufacturer's guidelines. NRO RNA fraction was immuno-purified using anti-Bromodeoxyuridine agarose conjugate IgG (Santa Cruz Biotechnology). Agarose beads were blocked in IP buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 0.05% NP40, 1 mM EDTA, 1× complete protease inhibitor) containing 0.1% polyvinylpyrrolidone and 1 mg/ml ultrapure BSA (Ambion). NRO-RNAs were added to 50 ml beads in 500 ml of IP buffer containing 1 U/μl of RNasin (Promega), and allowed to bind 1 h. The beads were washed in IP buffer five times. RNA was extracted using TRIzol reagent (Invitrogen) and precipitated. Descriptions of the steps of NRO assay as well as estimation of purity of NRO RNA fraction are done in Supplementary Figures S1–S3 and Supplementary 'Materials and Methods' section. Three to six independent NRO experiments were done for every genotype.

ChIP

A total of ~100 pairs of ovaries were dissected for every IP reaction. ChIP was performed according to the published procedure (38). Chromatin was immunoprecipitated with the following antibodies: anti-dimethyl-histone H3 Lys4 (Upstate) and anti-dimethyl-histone H3 Lys79 (Millipore). Quantitative PCR was conducted on DT-96 machine from DNA Technology, Russia. Quantities of target genomic regions precipitated by different antibodies were normalized to a fragment of transcriptionally inactive intergenic spacer in the 60D region. The ratio of these values calculated for homozygous and heterozygous *spn-E* mutants or *si_piwi* and control *w^1118* flies represents at the histograms. Eight serial 3-fold dilutions of input DNA of corresponding line were amplified in duplicates with each primer pair to make a standard curves. Standard deviation of duplicate PCR measurements for two independent ChIP experiments was calculated to determine the error of measurement. *rp49* gene was included in the analysis as positive control for transcriptionally active chromatin. Levels of H3K79me2 and H3K4me2 marks at *rp49* are similar in *spn-E*/+ and *spn-E*/*spn-E* mutants as well as in *w^1118* and *si_piwi* flies and display 30- to 80-fold enrichment compared to 60D intergenic spacer in different ChIP assays (data not shown). Normalization of ChIP and NRO data on the retrotransposon copy number was done using ChIP input data for each genotype. No substantial differences in the retrotransposon copy number were observed between *spn-E*/+ and *spn-E*/*spn-E* or *w^1118* and *si_ piwi* flies.

RESULTS

HeT-A/*yellow* endogenous reporter gene is a target for the piRNA-mediated silencing

Broken chromosome ends, as well as normal chromosomes, can be elongated by attachments of telomeric retroelements. We used *Drosophila* lines with *HeT-A* attachments to a truncated X chromosome with a break in the *yellow* locus to study expression of an endogenous reporter *yellow* gene in the piRNA pathway mutants. If the *HeT-A* attachment occurred after degradation of the *yellow* promoter, the *yellow* gene is transcribed from the *HeT-A* promoter located at its 3′ region (39,40). This results in a yellow-to-black change in aristae pigmentation which serves as a marker of terminal attachment events.

Yellow expression was analyzed in three independent lines with *HeT-A* attachments at different positions of the degraded *yellow* promoter region in the presence of piRNA pathway gene mutations. In such lines, a *HeT-A*/*yellow* fusion transcript is formed. Here, we present data for *z2* line, in which *HeT-A* attachment has occurred 50-bp downstream of the *yellow* transcription start site. The 5′ RACE analysis using *yellow*-specific primers revealed *HeT-A*/*yellow* 5′-ends at positions −30- and −95-bp upstream of the *HeT-A* polyadenylation site in the *z2* line. A major transcription site was mapped to position −30 because transcription at the −30 position is initiated about ten times more efficiently than that at the

−95 position (Supplementary Figure S4). The RT–PCR analysis revealed that the abundance of *HeT-A/yellow* transcripts is under control of the piRNA silencing pathway components Spn-E, Aub and a product of the *vasa* locus (Figure 1); it is several times more abundant in the ovaries of transheterozygous mutants as compared with heterozygous flies. It should be noted, that we obtained similar effects for all studied lines with the *HeT-A* attachment to the truncated *yellow* (data not shown).

In *Drosophila*, chromosome ends are capped by a protein complex including HP1 (41,42). Despite the absence of telomeric retrotransposon sequences at terminally deleted chromosomes, HP1 binds the terminus of these chromosomes in salivary glands, thus providing the chromosome capping (41,43). An extensive co-localization between a signal from anti-HP1 antibodies with the *yellow* and *HeT-A* staining is seen on ovarian nurse cell chromosomes of the *z2* line. Thus, HP1, a main component of the cap, is associated with the normal chromosome ends as well as with broken X chromosome in ovaries (Figure 1C). This fact allows us to consider that *HeT-A/yellow* reporter localizes in a protein environment similar to that of endogenous chromosome ends.

Expression of the *HeT-A/lacZ* transgenic construct is upregulated in the ovaries of piRNA pathway mutants

To address how the chromatin context affects the piRNA-mediated transgene silencing, we have studied the expression of a reporter gene driven by the *HeT-A* promoter and located in euchromatin, in the ovaries of piRNA pathway mutants. A construct containing a 3′-UTR fragment of the *HeT-A* copy derived from the *z2* line, fused to *lacZ* was used to generate transgenic flies (Supplementary 'Materials and Methods' section). The expression of transgenic β-galactosidase was studied in the ovaries of piRNA pathway mutants. We observed derepression of the transgene in the ovaries of *spn-E* and *piwi* mutants in three independent lines containing the transgene at different euchromatic sites (Figure 2B and C). Increase of the β-galactosidase activity in *spn-E* mutants correlates with an elevated level of *HeT-A/lacZ* RNA according to the RT–PCR analysis (Supplementary Figure S5).

It is noteworthy, that expression pattern of the reporter gene reproduces the expression patterns of endogenous telomeric *HeT-A* elements in the ovaries. Figure 2 shows distribution of endogenous *HeT-A* transcripts in the ovaries of wild-type flies, *piwi/+* and *piwi/piwi* females in comparison with the transgenic β-galactosidase staining in the flies of the same genotypes. No staining was observed in the wild-type ovaries. In heterozygous *piwi* mutants, *lacZ* expression is detected in the germarium, which is similar to the distribution of endogenous *HeT-A* transcripts (Figure 2C). Immunostaining of β-galactosidase detects it in the germinal cysts of *piwi/+* females (Supplementary Figure S6). Thus, the tissue-specific expression of the transgene in ovaries is regulated by the *HeT-A* promoter region which is present in the construct. Using 5′ RACE analysis, we have shown that a fused *HeT-A/lacZ* transcript contains ∼30 nt of the *HeT-A* 3′
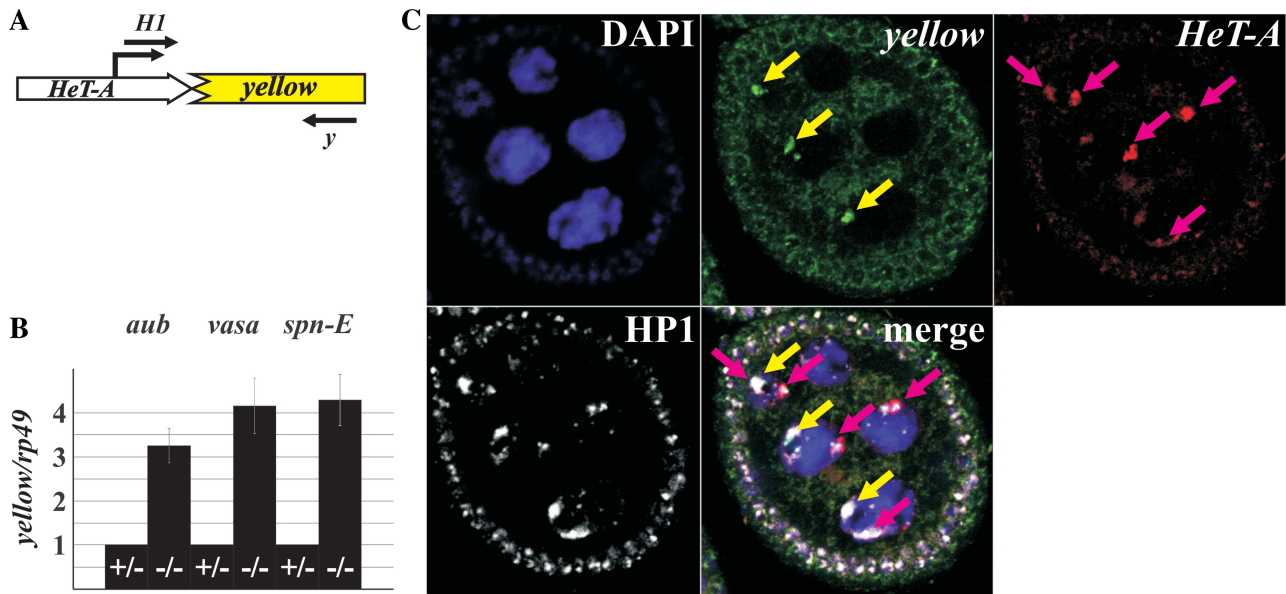

Figure 1. *HeT-A/yellow* reporter gene expression in the piRNA pathway mutants. (**A**) A schematic representation of the *HeT-A/yellow* reporter construct. Position of the transcription start site is indicated by broken arrow. Primers used in the RT–PCR analysis are shown. (**B**) RT–qPCR analysis of the *HeT-A/yellow* transcript amount in the ovaries of *spn-E, aub, and vasa* mutants. Bars of histograms represent a ratio of *HeT-A/yellow* to *rp49* transcript abundance in the ovaries of transheterozygous *aub^{QC42}/aub^{HN}* (−/−), *spn-E^1/spn-E^{hls3987}* (−/−), or *vig^{EP812}/vas^{PH165}* (−/−) flies related to this ratio in *aub^{QC42}/CyO* (+/−), *spn-E^1/TM3* (+/−), or *vig^{EP812}/CyO* (+/−) females. (**C**) Terminally deleted chromosome recruits the telomere capping protein HP1 in ovaries. Immunostaining of HP1 (white) reveals numerous signals in the ovarian nuclei. DNA FISH was performed with *yellow* (green signals indicated by yellow arrows) or *HeT-A* (red signals indicated by pink arrows) DNA probes. HP1 locates at the ends of normal chromosomes (*HeT-A* staining) as well as of terminally deleted chromosome (*yellow* staining) of the *z2* line. DNA is stained with DAPI (blue).
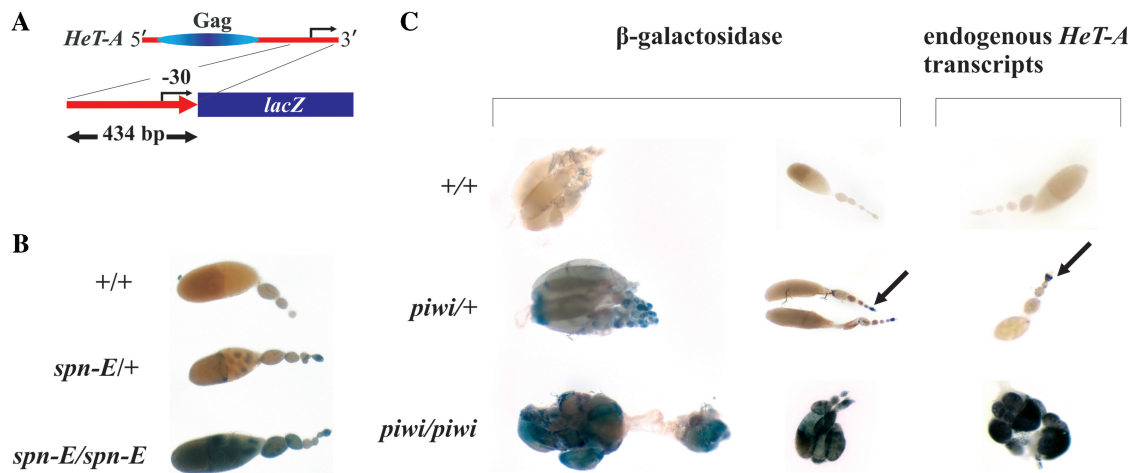
Figure 2. *HeT-A/lacZ* reporter gene expression in the piRNA mutants. (**A**) A schematic representation of the *HeT-A/lacZ* reporter construct. Position of the transcription start site is indicated by arrow. (**B**) X-gal staining of the β-galactosidase in the ovaries of *Df (1)w$^{67c23(2)}$*, *y* (+/+), *spn-E^1/TM3* (*spn-E/+*) and *spn-E^1/spn-E^{hls3987}* (*spn-E/spn-E*) flies bearing *HeT-A/lacZ* transgene (H95 line). Identical results were obtained for H12 and H124 transgenic lines. (**C**) Comparison of the endogenous *HeT-A* transcript localization (on the right) and β-galactosidase staining (on the left) in the ovaries of *Df (1)w$^{67c23(2)}$*, *y* (+/+), *piwi^2/CyO* (piwi/+), *piwi^2/piwi^3* (piwi/piwi) flies bearing *HeT-A/lacZ* transgene (H12 line). Arrows indicate staining in the germarium.

region as a result of the presence of transcription initiation site in the *HeT-A* 3′-UTR (Supplementary Figure S4). We failed to detect a second transcription start site, possibly due to the absence of some regulatory elements in the construct which provide a proper initiation of transcription. Thus, expression of the reporter genes driven by the *HeT-A* promoter is affected by the piRNA silencing pathway independent of the reporter gene location, in euchromatin or in telomeric heterochromatin.

Transcriptional analysis

Previously, we demonstrated that abundance of telomeric element transcripts of both directions is regulated by the piRNA pathway. *HeT-A* and *TART* transcripts of both polarities accumulate in the germ cell nuclei of homozygous piRNA pathway mutants (27). *HeT-A* and *TART* transcript hybridization signals in nuclei look like distinct bright dots, which may reflect the accumulation of nascent transcripts at the sites of transcription. To verify this assumption we performed a combined RNA and DNA localization in ovaries using RNA and DNA FISH analysis (Supplementary 'Materials and Methods' section). *HeT-A* sense and *TART* antisense transcripts were shown to be accumulated at the telomeric loci in *spn-E* mutants (Supplementary Figure S7). This observation argues in favor of a transcriptional or co-transcriptional mechanism of the piRNA-based expression regulation of endogenous telomeric elements.

To directly determine whether the piRNAs induce transcriptional silencing of the telomeric retrotransposons, we performed NRO assay. This method is capable of estimating the density of transcriptionally active RNA–polymerase complexes at the locus of interest (44,45). Retrotransposons were shown to be transcribed by RNA polymerase II (46). To isolate fractions of NRO RNA, we performed run-on reaction in the presence of BrUTP followed by immunoprecipitation and purification of

BrUTP-labeled nascent RNA (Supplementary Figure S3). NRO RNA was reverse transcribed and used for qPCR analysis. *rp49* served as an internal control for the normalization of transcription level values. We estimated transcription levels of endogenous and transgenic telomeric elements in piRNA pathway gene *spn-E* mutants. The *H95* transgenic line and *z2* line bearing *HeT-A* attachment to the broken X-chromosome were used for analysis. For both lines, transcription level of endogenous *HeT-A* and *TART* elements was significantly higher in the *spn-E/spn-E* ovaries than in *spn-E/+* ones (Figure 3A and B). *HeT-A/lacZ* and *HeT-A/yellow* transcription levels also increased in the ovaries of *spn-E* mutants (Figure 3A and B). These data indicate that telomeric retrotransposons and reporter constructs bearing *HeT-A* promoter undergo piRNA-mediated transcriptional silencing.

PIWI is a crucial component of piRNA pathway. Its mutations lead to a drastic ovary degeneration impeding experiments with *piwi* mutants. We applied RNAi approach to repress *piwi* expression in the germline (see 'Materials and Methods' section). In *si_piwi* flies PIWI was detected only in the follicular cells whereas ovarian germ cells lacked PIWI (Supplementary Figure S8). These flies are sterile in spite of morphologically normal ovary development. RT–PCR analysis demonstrates derepression of different retrotransposons in the *si_piwi* ovaries (Supplementary Figure S9). *piwi* knockdown results in the *HeT-A* and *TART* retrotransposon transcript accumulation in the ovaries (Supplementary Figure S10). The pattern of their distribution is similar to that in the other piRNA pathway mutants (28,34). *HeT-A* piRNAs are not detected in the ovaries of *si_piwi* flies (Supplementary Figure S10). NRO assay of the *si_piwi* ovaries reveals activation of endogenous *HeT-A* and *TART* transcription (Figure 3C).

Thus, piRNA-mediated telomeric retrotransposon silencing is in part transcriptional. Is this phenomenon
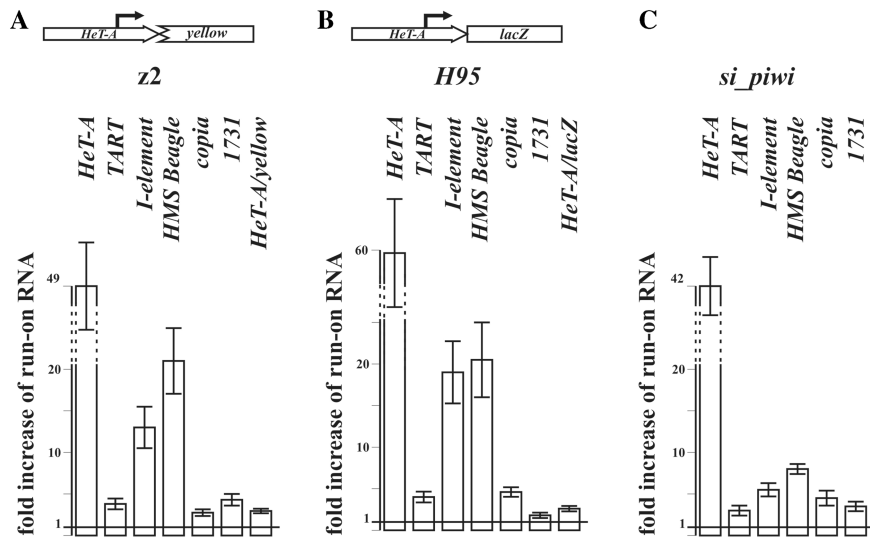
Figure 3. NRO analysis of the retrotransposon transcription in the *spn-E* mutants and in the *piwi*-knockdown flies. NRO analysis of transcription of the endogenous retrotransposons and reporter genes in the ovaries of *z2* line (**A**) and *H95* line (**B**) bearing *spn-E* mutations. A schematic representation of the reporter construct is given above the diagram. NRO analysis of the endogenous retrotransposon transcription in the ovaries of *si_piwi* flies (**C**). Bars of histograms represent a ratio of genes to be tested to *rp49* transcript abundance in the *spn-E¹/spn-E^{hls3987}* or *si_piwi* flies related to this ratio in *spn-E¹/TM3* or *w^{1118}*, respectively.

universal for the retrotransposon silencing in the germline? NRO assay of *spn-E* mutants and *si_piwi* ovaries reveals accumulation of run-on transcripts of *I*-element, *HMS Beagle, 1731* and *copia* retrotransposons, which were described previously as targets of the piRNA-mediated silencing (14,34,47) (Supplementary Figure S9 and Figure 3).

Taking into account the result of NRO assay, we examined the presence of transcriptionally active chromatin marks on the retrotransposon sequences in the piRNA pathway mutants. We focused on the two modifications of histone H3, dimethylation of lysine 79 (H3K79me2) and dimethylation of lysine 4 (H3K4me2). These two histone modifications were previously reported to be tightly linked to RNA polymerase II activity and correlate with the level of transcription (48). We performed ChIP experiments of chromatin of ovarian tissues comparing *spn-E¹/spn-E^{hls3987}* mutants to *spn-E¹/+* heterozygous flies. We observed enrichment of H3K79me2 and H3K4me2 transcription elongation marks within *HeT-A, TART, HMS Beagle* and *I*-element sequences in the ovaries of *spn-E¹/spn-E^{hls3987}* (Figure 4A and B). Data concerning *copia* and *1731* expression are ambiguous. These retrotransposons express not only in the germline but also in follicular cells at a substantial level (data not shown). This could mask transcriptional effects (if any) occurring in the germline. ChIP experiments performed in ovaries of *si_piwi* flies show increase of both histone marks at the retrotransposon chromatin compared to control *w^{1118}* line (Figure 4C). *HeT-A/lacZ* and *HeT-A/yellow* transgenes are also enriched by H3K79me2 and H3K4me2 marks in the *H95* and *z2* lines, respectively, bearing *spn-E* mutations (Figure 4A and B). ChIP analysis was done for *T6* line bearing *spn-E* mutations. In this line, *HeT-A* attachment to the truncated X chromosome has occurred 24-bp upstream of the *yellow* transcription start site just

downstream of the TATA-box. The data obtained are in accord with the results obtaining for the *z2* line corroborating the transcriptional activation of the endogenous retrotransposons and *HeT-A/yellow* reporter in the ovaries of the *spn-E* mutants (data not shown).

Taken together, these data strongly suggest a transcription activation of retrotransposons in the germline of piRNA pathway mutants.

DISCUSSION

In this study, we have explored different approaches to reach understanding of the mechanism of the piRNA-mediated silencing in the germline. The main objects of our study are telomeric retroelements which are located at the telomere ends in a special environment of the telomeric chromatin.

A remarkable feature of the *HeT-A* promoter is its ability to reproduce an expression pattern characteristic of the endogenous promoter while being located in ectopic positions of the genome. The *HeT-A* promoter constructs were shown to be active in the *Drosophila* somatic tissues (49). We have shown a similar expression pattern of endogenous telomeric *HeT-A* and euchromatic *HeT-A/lacZ* transgenes in the germline. Thus, ∼400 bp of the *HeT-A* promoter is enough to provide tissue specificity in its expression.

We have shown that both *yellow* and *lacZ* reporter genes, located in telomeric region or in euchromatin, respectively, demonstrated the dependence of their expression on the piRNA pathway being transcribed from the *HeT-A* promoter. Fusion *HeT-A/lacZ* and *HeT-A/yellow* transcripts contain 30–95 bp of the *HeT-A* sequence that may be enough for homologous recognition and post-transcriptional degradation the fusion transcripts by the piRNA-mediated machinery. *HeT-A*-specific piRNAs
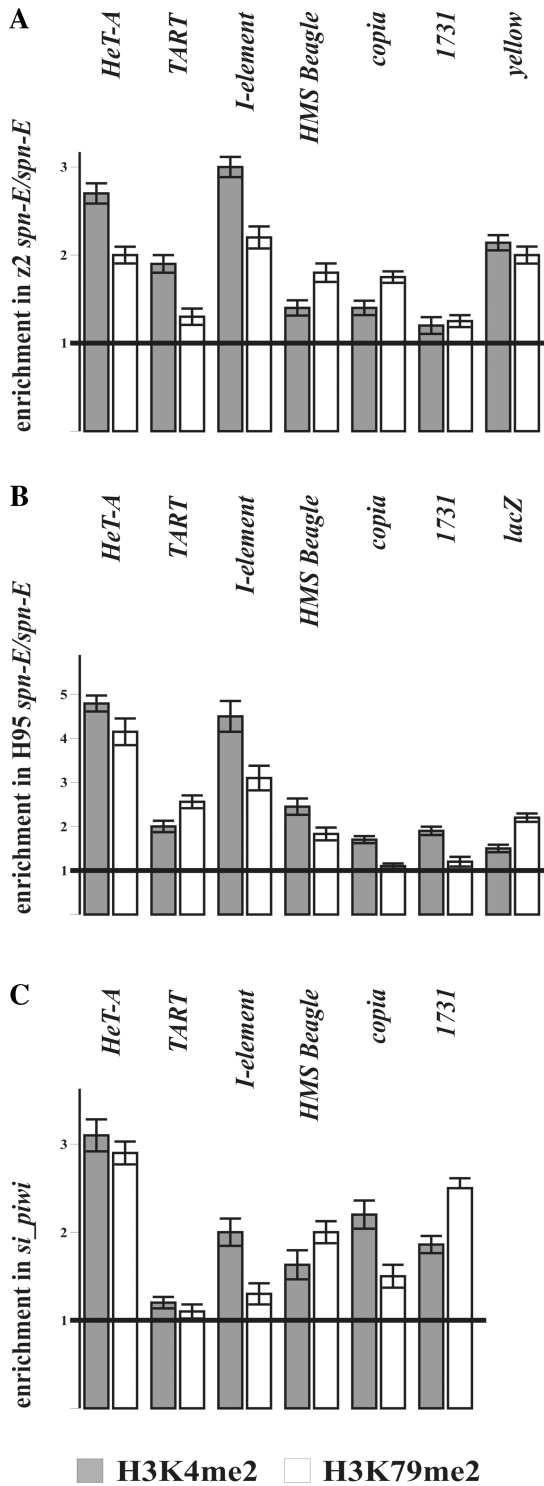
Figure 4. The level of transcriptionally active chromatin marks increases at the retrotransposon sequences in the ovaries of piRNA pathway mutants. ChIP of retrotransposon and transgene sequences in the *z2* line (**A**) and *H95* transgenic line (**B**) bearing *spn-E* mutations and in RNAi *piwi* ovaries (**C**) with antibodies specific to H3K79me2 (white) and H3K4me2 (gray). Amount of the PCR product was normalized to that of 60D intergenic spacer for each primer set. Ratio of these values for transheterozygous *spn-E¹/spn-Ehls3987* to heterozygous *spn-E¹/+* mutants (A and B) or *si_piwi* to *w¹¹¹⁸* flies (C) is shown on histograms. Error bars indicate standard deviation of duplicate PCR measurements for two independent ChIP experiments.

complementary to the 3′ *HeT-A* fragment cloned in the *HeT-A/lacZ* construct are represented in the piRNA libraries (47). Their amount is strongly drops in *piwi/piwi* and *spn-E/spn-E* mutant ovaries (Supplementary Figure S11A). More interesting is the presence of the pull of piRNAs complementary to the very 3′ *HeT-A* end which is a part of the *HeT-A-lacZ* and *HeT-A/yellow* fusion transcripts (downstream of −30 transcription start site) (Supplementary Figure S11B). These piRNAs may recognize the fusion transcript resulting in the post-transcriptional degradation. According to another scenario, the same piRNAs may target a nascent transcript and transmit a signal to chromatin exerting transcriptional silencing.

The NRO assay confirmed by ChIP analysis reveals activation of endogenous *HeT-A* and *TART* transcription in *spn-E* mutants and in flies with *piwi* knockdown in the female germline. Most pronounced effect was observed for *HeT-A* retrotransposon. The accumulation of nascent *HeT-A* and *TART* transcripts at the telomeres of piRNA mutants also indicates a transcriptional or co-transcriptional mode of the regulation of their expression. Both reporter constructs bearing *HeT-A* promoter undergo piRNA-mediated transcriptional silencing independent of the construct location, in euchromatin or at the chromosome end. Transcriptional mechanism seems to be universal for the piRNA-mediated retrotransposon silencing in the germline. We detect transcriptional activation of *I*-element and *HMS Beagle* retrotransposons in *spn-E* mutants and in flies with *piwi* RNAi knockdown. Most likely, both post-transcriptional and transcriptional pathways may be involved in the piRNA-mediated silencing of the same target gene, although it is difficult to estimate quantitatively contribution of each mechanism in the total silencing effect. Deep sequencing of the run-on cDNA library and ChIP-seq analysis may be helpful to obtain more detailed information about targets of the piRNA-mediated transcriptional silencing.

A siRNA-mediated spreading of heterochromatin was reported to be involved in the centromeric and telomeric repeat silencing in yeast. We tested the *Drosophila* lines in which the attachments of *HeT-A/TART* occurred upstream of the intact *yellow* regulatory region to the terminal deleted X chromosome, at a distance of ∼100–1000 bp from the *yellow* transcription start site. In these cases, no changes of the *yellow* expression were observed in *spn-E, aub* and *vasa* mutants (data not shown). This fact indicates that the inactive chromatin, if to assume its formation at the *HeT-A/TART* retrotransposon sequences, does not spread onto the adjacent *yellow* promoter region. Indeed, the terminal retrotransposon array exhibits euchromatic characteristics since P-element insertions into this retrotransposon array are not silenced (50). Telomeric retrotransposon repeats display the features of open chromatin, however, no actively elongating RNA polymerase isoforms were detected in this region (51). Components of the transcriptional initiation complex of the retrotransposons may be considered as a putative link between piRNAs and inhibition of the transcription.

The *aub* and *armi* piRNA pathway gene mutations were shown to affect telomere capping protein recruitment (52)

which may result in the telomeric retrotransposon overexpression. However, we show that transcription of non-telomeric retrotransposons as well as *HeT-A/lacZ* euchromatic transgene is activated as a result of the piRNA pathway disruption. This suggests that piRNA-mediated transcriptional silencing is rather universal phenomenon and not restricted to the specific telomeric region which is affected by the telomere capping. We do not exclude the possibility that genomic context influences the piRNA-mediated chromatin protein recruitment to the target sequences. This may be the case in the telomeric retrotransposon array as well. Transcriptional silencing complexes may be distinct on the distal-most telomeric retroelements covered by the capping proteins and on the telomeric retroelements located proximally.

Transposable element families consist of heterogeneous copies some of which are active and located in euchromatin, others are represented by damaged heterochromatic elements. It is clear that such transposon subfamilies have principally different regulatory mechanisms and chromatin structure. Most experimental approaches fail to distinguish between different types of transposons which result in the complicated superposition of distinct effects. An important result of this study is that unique transgene containing *HeT-A* promoter exhibits expression pattern and piRNA-mediated transcriptional silencing similar to the endogenous telomeric *HeT-A*. The transgenic system might be an important tool in the understanding of the piRNA-mediated transcriptional silencing mechanism in the germline.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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