268–278 *Nucleic Acids Research, 2009, Vol. 37, No. 1* *Published online 26 November 2008*
doi:10.1093/nar/gkn960

rasiRNA pathway controls antisense expression of *Drosophila* telomeric retrotransposons in the nucleus

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Received July 9, 2008; Revised November 10, 2008; Accepted November 12, 2008

ABSTRACT

Telomeres in *Drosophila* are maintained by the specialized telomeric retrotransposons *HeT-A*, *TART* and *TAHRE*. Sense transcripts of telomeric retroelements were shown to be the targets of a specialized RNA-interference mechanism, a repeat-associated short interfering (rasi)RNA-mediated system. Antisense rasiRNAs play a key role in this mechanism, highlighting the importance of antisense expression in retrotransposon silencing. Previously, bidirectional transcription was reported for the telomeric element *TART*. Here, we show that *HeT-A* is also bidirectionally transcribed, and *HeT-A* antisense transcription in ovaries is regulated by a promoter localized within its 3′ untranslated region. A remarkable feature of noncoding *HeT-A* antisense transcripts is the presence of multiple introns. We demonstrate that sense and antisense *HeT-A*-specific rasiRNAs are present in the same tissue, indicating that transcripts of both directions may be considered as natural targets of the rasiRNA pathway. We found that the expression of antisense transcripts of telomeric elements is regulated by the RNA silencing machinery, suggesting rasiRNA-mediated interplay between sense and antisense transcripts in the cell. Finally, this regulation occurs in the nucleus since disruption of the rasiRNA pathway leads to an accumulation of *TART* and *HeT-A* transcripts in germ cell nuclei.

INTRODUCTION

RNA interference (RNAi) evolved as a defense mechanism against viruses that can generate double-stranded RNA (dsRNA). Long dsRNA is processed by a ribonuclease enzyme, Dicer, into small interfering RNAs (siRNAs), guiding degradation of homologous mRNA (1). This mechanism was adapted to control the expression and transposition of endogenous transposable elements in different organisms (2–12). A distinct class of short RNAs, repeat associated short interfering RNAs (rasiRNAs), 24–27 nt long, is involved in the silencing of retrotransposons in the *Drosophila* germ line (13). Importantly, rasiRNA production does not require processing of a dsRNA precursor by Dicer, a key enzyme for microRNA and siRNA production (13). Sense and antisense rasiRNAs specific to different classes of retrotransposons, long terminal repeat (LTR) and non-LTR elements are revealed in libraries of short RNAs (12,14) and by northern analysis (15,16). Although the mechanism of rasiRNA processing still remains unclear, it is obvious that antisense transcripts are important participants in this process.

First of all, antisense transcripts of transposable elements serve as a source for rasiRNA generation. A certain amount of rasiRNA is produced from heterochromatic loci enriched in damaged inactive copies of transposable elements (11,12). These small RNAs are believed to originate from a putative long, single-stranded precursor. The *flamenco*/*COM* locus has been shown to regulate the activity of the retroviral *gypsy* element (17) and two other retroelements, *Idefix* and *ZAM* (18). rasiRNAs originating from the *flamenco* locus are proposed to control the activities of *Idefix, ZAM* and *gypsy* retrotransposons in the *Drosophila* germline (12). Similar orientation of the copies of these elements in the *flamenco* locus results in the production of mainly antisense rasiRNAs. Some other described *Drosophila* and mouse loci generate small RNAs from both strands as a result of mixed orientations of transposable elements within the loci (11,12). Primary short RNAs guide the generation of additional short RNAs of both polarities. According to the 'ping-pong' amplification model, sense rasiRNAs result from the processing of long sense transcripts with the assistance of PIWI- or Aubergine (Aub)-associated antisense rasiRNAs and sense rasiRNAs in the complex with

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Ago3 guide the cleavage of antisense transcripts to produce additional antisense rasiRNAs (12,19). Being either the primary source of rasiRNA production or the template for subsequent amplification, antisense transcripts are apparently necessary for the RNA silencing process.

Antisense transcripts of transposable elements can be generated in different ways. They may be derived from read-through transcription from an adjacent external promoter, which has been documented, for example, in the *Caenorhabditis elegans* transposon Tc1 (4). Several examples of retrotransposon antisense internal promoters were described, although their functional significance is unclear. A testis-specific antisense promoter of the LTR retrotransposon *micropia* in *Drosophila hydei* drives expression of the transcript complementary to the reverse transcriptase open reading frame (ORF) (20). Antisense promoters located in the 5′ untranslated region (UTR) downstream of the sense transcription start site are found in the human non-LTR elements L1 (21–23) and the *Drosophila* F-element (24). Antisense L1-specific short RNAs complementary to the region of overlapping transcripts were shown to contribute significantly to the RNAi-mediated silencing of L1 (25). Additionally, the *Chlamydomonas reinhardii* element TOC1 (26), the *Tripanosoma brucei* retroelement TRS (27) and the *Dictyostelium discoideum* retroelement DRE (28) are transcribed in both directions. These data suggest multiple sources for transposon-specific antisense RNA origination.

The *HeT-A*, *TART* and *TAHRE* families of *Drosophila* non-LTR retrotransposons play important roles in the cell, providing telomere repair (29–32). 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. These elements are found at *Drosophila* telomeres in mixed tandem head-to-tail arrays; their 3′-ends are orientated toward the centromere. Telomeric elements are characterized by the presence of long 3′- and 5′-UTRs and the unusual structure of their promoters. Most of the non-LTR retrotransposons utilize an internal promoter in order to transcribe a full-length RNA that serves as a template for transposition according to the target-primed reverse transcription mechanism (33–35). Promoters of *HeT-A* and *TAHRE* are localized in the 3′-UTR and drive transcription of a downstream element (32,36). The *TART* element was shown to be transcribed bidirectionally from internal sense and antisense promoters that were localized within nonterminal direct repeats in the *TART* 5′- and 3′-regions (37,38). Interestingly, *TART* antisense transcripts are spliced (38). The abundance of telomeric retroelement transcripts and the frequency of their transpositions onto chromosome ends are controlled by an rasiRNA-mediated mechanism (10,32). Nothing is yet known about the origin of antisense transcripts of the main structural telomeric element, *HeT-A*. Only sense *HeT-A* transcription was observed by northern (37) or *in situ* RNA hybridization analyses (10,39), while *HeT-A* antisense rasiRNAs were revealed to be among the cloned short RNA species in *Drosophila* (12,14) and by northern analysis (10,13).

Taking into account a crucial role of RNA silencing in the regulation of the telomeric element expression in the *Drosophila* germline (10), we studied *HeT-A* antisense expression as a putative source of antisense rasiRNAs in the ovaries. We mapped the antisense transcription start site to the 3′-UTR of this element. Multiple introns are present in the noncoding, antisense *HeT-A* transcripts, and their hypothetical significance is discussed.

Increasing numbers of natural sense–antisense transcript pairs are being identified in a variety of eukaryotic organisms (40–44). Experimental evidence suggests that some antisense transcripts may be involved in repression of the gene expression via natural RNAi (45–48). However, biogenesis of antisense RNAs is poorly understood. Endogenous antisense transcripts have never been considered as a natural target of the RNAi-based mechanism. Investigations are usually focused on the coding, sense transcripts, which, in the case of transposable elements, serve as a template for transpositions. However, their abundance is regulated by antisense short RNAs derived from long antisense transcripts. Here, we have shown that natural antisense transcripts, like the sense ones, are targets of the RNAi machinery. The abundance of antisense transcripts of *Drosophila* telomeric transposable elements is affected by mutations of the rasiRNA pathway genes. Thus, overall expression level of *HeT-A* and *TART* retrotransposons could be a result of a complex rasiRNA-mediated interplay of their sense and antisense transcripts. Our data demonstrate that this regulation occurs in the nucleus since the disruption of the rasiRNA pathway leads to the accumulation of *TART* and *HeT-A* transcripts in the germ cell nuclei in *Drosophila*.

MATERIALS AND METHODS

Drosophila strains

Strains bearing *spindle-E* (*spn-E*) mutations were ru^1 st^1 $spn\text{-}E^1$ e^1 $ca^1/TM3$, Sb^1 e^s and ru^1 st^1 $spn\text{-}E^{hls3987}$ 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 (49,50). We used $piwi^2$ and $piwi^3$ mutations and *Drosophila melanogaster* stock y^1; cn^1 bw^1 sp^1 (Bloomington *Drosophila* stock center #2057).

Northern analysis of short RNAs

Northern analysis of short RNAs was done as previously described (8). Ovarian RNAs enriched with short RNAs were isolated using the miRACLE miRNA isolation kit (Stratagene). P^{32}-labeled riboprobes corresponding to sense or antisense strands of *TART* and *HeT-A* were synthesized. PCR fragments of retrotransposons cloned into pBS SK^- were used as transcription templates. The *TART* probe contained a fragment of the ORF2 corresponding to nucleotides 2377–2888 of the GenBank sequence DMU02279. The *HeT-A* probe contained a fragment of the 3′-UTR (nucleotides 6769–7092 of the GenBank sequence DMU06920). Hybridization with P^{32} end-labeled oligonucleotide 5′-ACTCGTCAAAATGGC TGTGATA-3′ complementary to *mir13b1* or 5′-TACA

ACCCTCAACCATATGTAGTCCAAGCA-3′ complementary to 2S RNA was used as a loading control.

In situ RNA hybridization and immunohistochemistry

In situ RNA analysis was carried out according to the previously described procedure (51) using digoxigenin (DIG)-labeled strand-specific *TART* and *HeT-A* riboprobes (described above) and rhodamine- (diluted at 1/500) or alkaline phosphatase (AP)-conjugated (diluted at 1/2000) anti-DIG antibodies (Roche). For *in situ* RNA hybridization using AP-conjugated anti-DIG antibodies, the time of incubation with AP substrate was more than 2 h. An RNaseH control was done to exclude the possibility of RNA–DNA hybridization in these experiments. After hybridization and washing, ovaries were treated with RNaseH (0.3 U/μl) for 1 h at 37°C followed by immunodetection. The *lacZ* fragment was PCR amplified using 5′-TTCCAGTTCAACATCAGCCGCTAC-3′ and 5′-GT TGATGTCATGTAGCCAAATCGG-3′ primers, was cloned in pBS SK$^-$, and used to obtain a *lacZ* riboprobe. The combination of protein and RNA localization is described in Supplementary Materials and Methods. To stain DNA, ovaries were incubated in PBS containing 0.5 μg/ml DAPI. The ovaries were visualized by confocal microscopy Zeiss LSM510 Meta.

5′ RACE analysis

RNA samples were prepared from the dissected ovaries of *D. melanogaster* stock y^1; cn^1 bw^1 sp^1 and from Schneider2 (S2) cells transfected by the HeTA-s or HeTA-as constructs ($\sim 7 \times 10^6$ cells) using TRIzol reagent (Invitrogen). RNA samples were treated with TURBO DNase (Ambion). 5′ RACE (rapid amplification of cDNA ends) analysis was performed using the 5′ RACE system (Invitrogen) according to the manufacturer's general guidelines. *HeT-A*-specific primers for the first strand cDNA synthesis and PCR (schematically represented in Figure 2) were: H-RT 5′-TACAATTTCCATGACGA CTC-3′ and H4 5′-GGAACCCATCTTCAGAATTCCC TC-3′ or M1 5′-CTGTCTCCGTACCTCCACCAGC-3′. Primers for the first strand cDNA synthesis and PCR, corresponding to the CaSpeR-AUG-β-gal sequence, were 5′-GAAAATCACGTTCTTGTTG-3′ and 5′-TGGT CAAAGTAAACGACATGGTGAC-3′, respectively.

RT-PCR analysis

Semi-quantitative RT-PCR was done according to the previously described procedure (8). RNA samples were divided for RT$^-$ and two RT$^+$ reactions. cDNA was synthesized using a random primer or gene-specific primers. Using *rp49* primers, amplification of the reverse transcription reaction obtained with a random primer was done as a loading control. It was observed that reverse transcriptase yields cDNA in the absence of primers, possibly as a result of unspecific priming. To avoid artifacts associated with this effect, we attached a universal adapter (5′-gcctgccccaacctcc-3′) to each gene-specific primer. A gene-specific part of such oligonucleotide serves as a primer for RT, and the adapter serves as a PCR primer. The results of the RT-PCR analysis were evaluated using the program ImageQuant5.0. Histograms display the quantification of at least three experiments.

The following primers were used (sequence of the adapter is given in bold):

rp49 (5′-ATGACCATCCGCCCAGCATAC-3′ and 5′-CTGCATGAGCAGGACCTCCAG-3′)
HeT-A asRT (5′-**GCCTGCCCCAACCTCC**GGAACC CATCTTCAGAATT-3′)
heT-A asPCR (5′-GCCAGAAGGACGGAAGCA CC-3′)
TART-A1 asRT (5′-**GCCTGCCCCAACCTCC**GATTG AGAGAGGTAAGAAG-3′)
TART-A1 asPCR (5′-GGAAACGAAGCAGAAAAA TCGC-3′)
HeT-A sRT (5′-**GCCTGCCCCAACCTCC**TGGAGTG CGTTGTCATAG-3′)
HeT-A sPCR (5′-GAAACACCAACAAAATACCCA AAAAGG-3′)
TART-A1 sRT (5′-**GCCTGCCCCAACCTCC**GAAG CAGAAAAATCGCG-3′)
TART-A1 sPCR (5′-CGATTGAGAGAGGTAAGAA GG-3′)

Analysis of promoter activity

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 (32). Clone z2 was used as a template to amplify 434 bp of the *HeT-A* promoter region. An *HeT-A* PCR fragment obtained with 5′-ATGA ATTCATCCATCGCCCGCAACATG-3′ and 5′-ATGG ATCCTTTGCTGGTGGAGGTACGGAGAC-3′ primers was inserted into the EcoRI and BamHI polylinker sites of CaSpeR-AUG-β-gal to obtain the HeTA-s construct. The *HeT-A* PCR fragment obtained with 5′-ATG AATTCTTTGCTGGTGGAGGTACGGAGAC-3′ and 5′-ATGGATCCATCCATCGCCCGCAACATG-3′ primers was inserted into the EcoRI and BamHI polylinker sites of CaSpeR-AUG-β-gal to obtain the HeTA-as construct. The constructs were verified by sequencing. *Drosophila* cell culture S2 was used for transfection, which was performed with FuGENE 6 Transfection Reagent (Roche) according to the manufacturer's recommendations. The β-galactosidase assay was done as previously described (52). Four experiments were done to evaluate the mean ± SD.

RESULTS

HeT-A antisense transcription start sites in ovaries

HeT-A antisense short RNAs were detected by northern analysis in *Drosophila* ovaries, using a probe corresponding to the ORF (10). In the ovarian short RNA libraries, antisense *HeT-A* rasiRNAs corresponding to 3′-UTR were also detected (12,14). Considering these data, we have proposed that antisense *HeT-A* RNA is transcribed from an unidentified promoter located at the 3′-UTR or just downstream. 5′ RACE analysis was applied to identify the *HeT-A* antisense transcription start site in *Drosophila* ovaries. Total RNA was extracted from

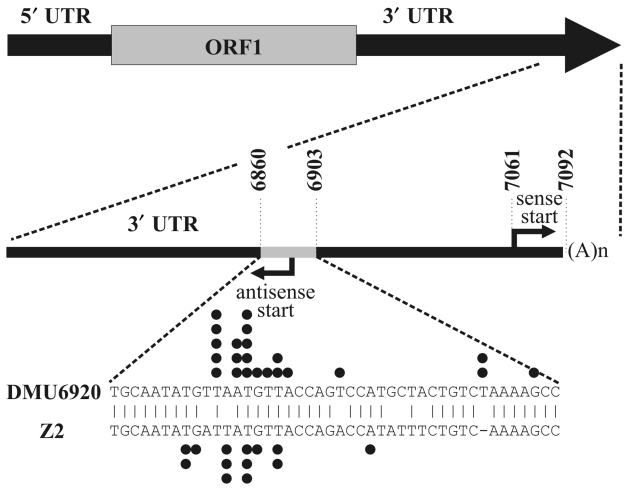

Figure 1. Positions of the *HeT-A* antisense transcription initiation sites identified by 5′ RACE. A schematic representation of the *HeT-A* element is given. Positions of the sense transcription start site and polyadenylation site are indicated in accordance with the sequence of the GenBank clone DMU6920. The upper sequence corresponds to position 6860–6903 in the clone DMU6920; the lower sequence represents a fragment of the *HeT-A* element z2 cloned in the reporter construct. The dot designates a single detected transcription initiation sites at the specific nucleotide, determined for endogenous *HeT-A*s (upper sequence) and for the HeTA-as construct in the transfection experiment.

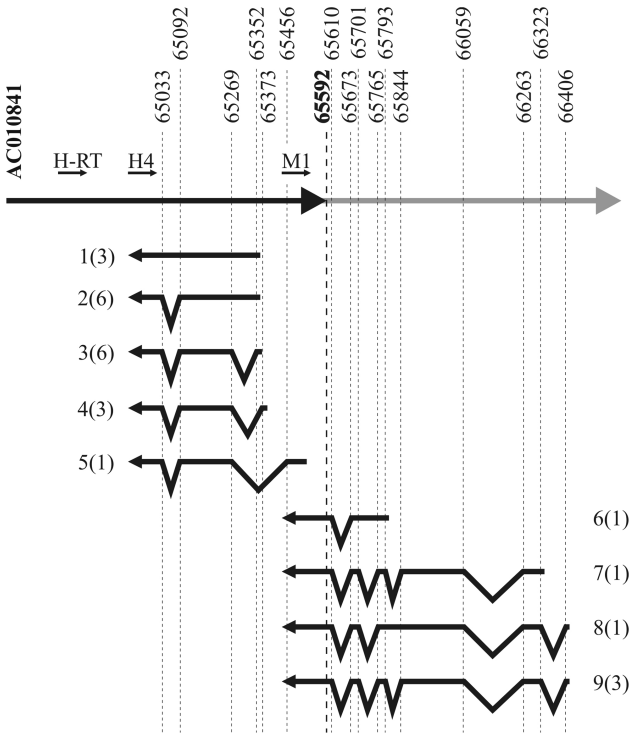

Figure 2. *HeT-A* antisense transcripts contain multiple introns. Structures of *HeT-A* antisense transcripts deduced from the sequences of 5′ RACE products. The fragment of the telomeric clone AC010841 containing the boundary between the two *HeT-A*s (designated by black and grey arrows) represents genomic *HeT-A* sequences. The primers used in the 5′ RACE analysis are indicated. Types of splicing are represented by arrows with schematically indicated introns and enumerated to the left (1–5) and right (6–9); the numbers in brackets are the numbers of independent analyzed 5′ RACE clones corresponding to the given scheme of splicing. Positions of the first and last nucleotides of the introns are indicated above the scheme in accordance with the sequence of clone AC010841. The number in bold is the boundary between two *HeT-A*s.

ovaries of the *D. melanogaster* stock y^1; cn^1 bw^1 sp^1, which was used in genome sequencing. 5′ RACE analysis yielded a number of PCR products. Heterogeneity of the 5′ RACE products results from the alternative splicing of antisense transcripts (described below). We have found that antisense transcription is initiated at heterogeneous sites within the region ~220 bp upstream of the polyadenylation site. Nucleotides 6870, 6872 and 6873 (according to *HeT-A* element 23Zn-1 from the genomic clone U06920, Figure 1) are frequently used as initiation sites of antisense transcription. The major antisense start site is located ~190 nt upstream of the *HeT-A* sense transcription start site (36). However, one of the 5′ RACE products starts as far as 30 bp upstream of the sense transcription start site (position 7029, according to *HeT-A* element 23Zn-1 from the genomic clone U06920).

In spite of the fact that primers used for 5′ RACE analysis match only a subset of *HeT-A* elements, the obtained products proved to be polymorphic, which allows us to conclude that antisense transcripts are derived from different elements. Most of them are highly homologous to *HeT-A* elements from the second and fourth chromosomes. For several other products we failed to determine the exact genomic origination. Analysis of 5′ RACE products that were obtained using a *HeT-A*-specific primer that anneals to the 3′-most end (Figure 2, primer M1) revealed that they had arisen from antisense transcription of the truncated *HeT-A* copy into an upstream *HeT-A* element. The sequence of such 5′ RACE products, including the boundary between *HeT-A* elements, aligns perfectly to the chromosome 4R telomeric clone (GenBank # AC010841; Figure 2), indicating that telomeric elements contribute to the overall level of *HeT-A* antisense expression. Thus, the antisense *HeT-A* promoter is localized at the 3′-UTR and is capable of driving read-through transcription from downstream into an upstream element in the telomere.

HeT-A antisense transcripts contain multiple introns

Sequencing of 25 5′ RACE clones revealed that most of the *HeT-A* antisense transcripts are spliced. Figure 2 shows structures of *HeT-A* antisense transcripts deduced from the sequences of 5′ RACE products. A fragment of the telomeric clone AC010841, containing the boundary between two *HeT-A*s, was chosen to represent a genomic *HeT-A* sequence (Figure 2). The right panel of Figure 2 (transcripts 6–9) shows read-through *HeT-A* antisense transcripts (described above) that were derived from these particular *HeT-A* elements. In these transcripts, different patterns of intron splicing are detected, suggesting the existence of alternative ways in which transcripts derived from a single element are processed. It is noteworthy, that other antisense *HeT-A* transcripts (transcripts 1–5, aligned to the left *HeT-A* copy in Figure 2) are heterogeneous and have different genomic origins. The *HeT-A* copy represented in Figure 2 has been

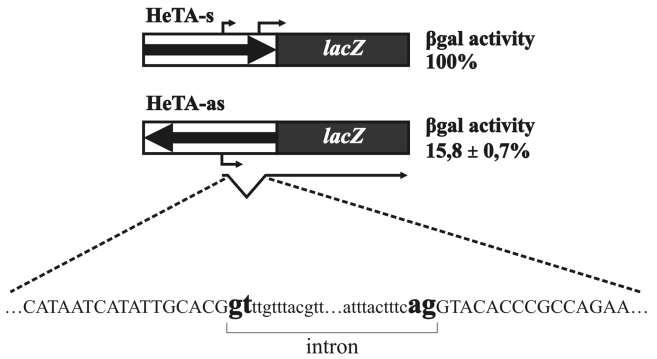

Figure 3. Antisense promoter activity of the *HeT-A* 3′-region. HeTA-s and HeTA-as constructs contain the *HeT-A* 3′ fragment cloned in sense and antisense orientation, respectively, in the pCaSpeR-AUG-β-gal vector. Enzymatic activity of the HeTA-as construct is expressed as a percentage of the activity of the HeTA-s construct. No enzymatic activity was detected in the cells transfected by the pCaSpeR-AUG-β-gal vector or in 'no DNA' control cells. Transcription start sites are indicated by short arrows. The *HeTA-as*/*lacZ* fusion transcript is indicated by a longer arrow and the intron is schematically designated. The sequence represents an exon and intron structure of the *HeT-A* genomic fragment deduced from the sequences of 5′ RACE products (exon and intron sequences are indicated by upper- and lowercase letters, respectively). The conserved 5′- and 3′-intronic boundary sequences (gt and ag) are in bold letters.

chosen for alignment as it is the most similar to transcripts 1–5 (Figure 2, left panel). Good matches to splice site consensus sequences are present at the exon/intron boundaries for all revealed introns in the *HeT-A* antisense transcripts. In some cases, intron sizes vary in different *HeT-A* copies (Figure 2). This was expected, taking into account the sequence polymorphism of *HeT-A* 3′-UTRs. Splicing in different polymorphic *HeT-A* copies is likely to occur at cryptic splice sites. Furthermore, introns were identified in the GenBank cDNA and EST clones, which correspond to *HeT-A* antisense transcripts (Supplementary Figure 1). *HeT-A* antisense transcripts are noncoding, as no long ORFs were found among these transcripts.

The 3′-end of *HeT-A* has antisense promoter activity

HeT-A sense promoter has been mapped to the 3′-end of the element (36). This promoter drives expression of a downstream element in a telomere. We have studied the antisense promoter activity of the *HeT-A* 3′-region, using transfection of cultured *Drosophila* cells by reporter constructs. A fragment of the *HeT-A* 3′-UTR containing 434 bp upstream of the polyadenylation site was cloned in both the sense and antisense orientations into the pCaSpeR-AUG-β-gal vector containing the *lacZ* reporter gene (HeTA-s and HeTA-as constructs, respectively, Figure 3). The enzyme activity was evaluated in extracts of transfected S2 cells. We detected low but reproducible reporter gene activity of the HeTA-as construct, which was evaluated at about 15% activity of the sense promoter, according to β-galactosidase activity assay (Figure 3). To determine the transcription start site in the HeTA-as construct, we performed the 5′ RACE analysis on the RNA isolated from S2 cells transfected by this construct. Primers corresponding to the *lacZ* reporter gene were used. We have found that transcription of the fused *HeT-A-lacZ* transcript initiates at heterogeneous start sites within region 217–235 nt upstream of the poly(A) site. Positions of antisense transcription start sites in the construct correspond closely to those determined for endogenous *HeT-A* copies (Figure 1). Also, like endogenous *HeT-A* antisense transcripts, transcripts from the HeTA-as construct are spliced (Figure 3). We performed 5′ RACE for the HeTA-s construct using the same conditions and found that, in this case, transcription is initiated at two major sites, 30–32 and 90–95 nt upstream of the *HeT-A* polyadenylation site (not shown). These data coincide well with the previously determined *HeT-A* transcription start sites (36,38) and confirm the reliability of the 5′ RACE data for *HeT-A* antisense transcription.

HeT-A rasiRNAs in RNAi mutants

Northern blot analysis was used to reveal *HeT-A*-specific rasiRNAs in the ovaries. Riboprobes containing sense or antisense strand sequences corresponding to the 3′-UTR were used. We detected *HeT-A* rasiRNAs of both polarities in the ovaries of $spn\text{-}E^{1}/TM3$, aub^{QC42}/Cy and $piwi^{2}/Cy$ flies. No sense or antisense *HeT-A* rasiRNAs were detected in the $spn\text{-}E^{1}/spn\text{-}E^{hls3987}$ and $piwi^{2}/piwi^{3}$ ovaries. A decreased amount of *HeT-A* rasiRNA was observed in aub^{QC42}/aub^{HN} ovaries (Figure 4).

In the ovarian short RNA libraries, both sense and antisense rasiRNAs were found for *HeT-A* and *TART* elements (12). The presence of the sense *HeT-A* and *TART* rasiRNAs suggests that the antisense transcripts are the targets of the RNAi machinery.

HeT-A and *TART* antisense expression in RNAi mutants

According to previously obtained data, sense *HeT-A* and *TART* transcripts accumulated in ovaries of the rasiRNA pathway component mutants (10,50), however, the patterns of their expression are different. *HeT-A* transcripts were detected both in oocyte and nurse cells, whereas *TART* transcripts substantially accumulated in nurse cells at the late stages of oogenesis. In parallel experiments, we failed to detect changes in the abundance and localization of *HeT-A* and *TART* antisense transcripts in the rasiRNA pathway mutants using *in situ* RNA hybridization with AP-conjugated anti-DIG antibodies (10). No sense and antisense *HeT-A* and *TART* transcripts were detected in the ovarian nuclei. However, *in situ* RNA hybridization using AP-conjugated anti-DIG antibodies and prolonged time of incubation with the AP substrate allowed us to reveal the accumulation of antisense *HeT-A* and *TART* transcripts in the ovaries with a compromised rasiRNA pathway (Figure 5, Supplementary Figure 2). Next, RNA fluorescence *in situ* hybridization (RNA FISH) was applied to compare cellular localization of sense and antisense *HeT-A* and *TART* transcripts in the ovaries of RNAi mutants.

Both *HeT-A* (Figure 5A) and *TART* (Figure 5B) antisense transcripts accumulated in the nuclei of nurse cells in the developing egg chambers of *spn-E*/*spn-E* mutants. Signals corresponding to the *TART* antisense transcripts are visible as bright dots in the nuclei of

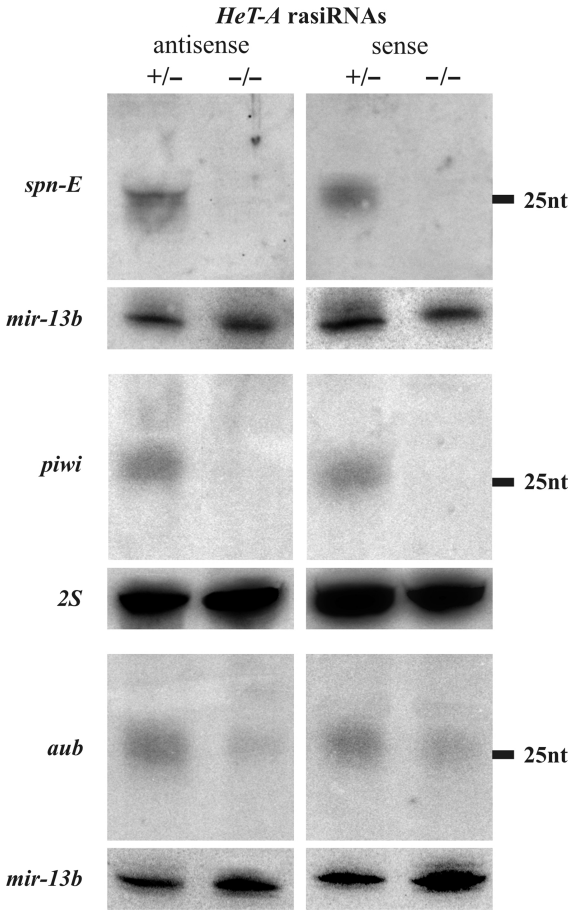

Figure 4. Effect of mutations of the rasiRNA pathway genes on the presence of sense and antisense *HeT-A*-specific rasiRNAs in ovaries. Northern analysis of the RNA isolated from ovaries of *spn-E*^{1}/*TM3*, *spn-E*^{1}/*spn-E*^{hls3987}, *piwi*^{2}/*CyO*, *piwi*^{2}/*piwi*^{3}, *aub*^{QC42}/*CyO* and *aub*^{QC42}/*aub*^{HN} flies. +/– and –/– designate hetero- and transheterozygous states of the corresponding mutations, respectively. Hybridization was done with *HeT-A* riboprobes from the *HeT-A* 3′-region to detect antisense (left part) or sense (right part) *HeT-A*-specific rasiRNAs. Lower panels represent hybridization with oligonucleotide complementary to the *mir-13b1* microRNA or 2S RNA. P^{33}-labeled RNA oligonucleotides were used as size markers.

nurse cells (Figure 5B). *HeT-A* antisense transcripts are found in nuclei and cytoplasm of the nurse cells in *spn-E* mutants (Figure 5A). No hybridization signal was detected with a probe for the *lacZ*, which indicates the specificity of hybridization (not shown). Accumulation of *TART* antisense transcripts in the nuclei of nurse cells of *aub* and *vasa* locus mutants was detected (Supplementary Figure 2).

As demonstrated previously, sense *HeT-A* transcripts are localized in the growing oocyte in rasiRNA pathway component mutants (10,50). We showed that, in *spn-E*/*spn-E* mutants, *HeT-A* sense transcripts are localized in the cytoplasm of the oocyte where the Orb protein is detected (Figure 5E). It is noteworthy that *HeT-A* and *TART* sense transcripts are accumulated not only in cytoplasm of germ cells as was shown previously, but also in nurse cell nuclei in *spn-E*/*spn-E* mutants (Figure 5C, D). *HeT-A* sense transcripts from nurse cells are most likely to be effectively transported to the oocyte. *TART* sense transcripts are accumulated in the nurse cell cytoplasm and nuclei at later stages of oogenesis and are not detected in oocytes of *spn-E*/*spn-E* mutants (Figure 5D). It is significant that hybridization conditions allowed us to detect RNA rather than genomic DNA. The treatment of ovaries by RNaseH after RNA hybridization did not affect the intensity of the nuclear signals (data not shown). Different patterns of hybridization with complementary sense and antisense single-stranded *TART* riboprobes is also a strong argument against the possibility of RNA–DNA hybridization. Actually, *TART* antisense transcripts are detected in nurse cell nuclei at different stages of oogenesis (Figure 5B), while sense transcripts are revealed in nuclei of nurse cells only at the late stage (Figure 5D).

The RT-PCR analysis of the cellular fractions of ovarian RNA was done to compare *HeT-A* and *TART* sense and antisense transcript abundance in *spn-E* mutants. The purity of cellular fractionation was determined by western and northern blot analysis (Supplementary Figure 3). Using RT-PCR with strand-specific primers, we detected substantial accumulation of *HeT-A* and *TART* antisense transcripts in the nuclear fraction of the ovarian RNA from *spn-E*/*spn-E* females (Figure 6). Sense transcript abundance in nuclei is also controlled by the *spn-E* gene, according to the RT-PCR analysis with strand-specific primers (Figure 6). These data confirm the accumulation of sense and antisense *HeT-A* and *TART* transcripts in ovarian nuclei of transheterozygous *spn-E* mutants obtained by RNA FISH analysis (Figure 5). The main difference between sense and antisense transcript behavior in RNAi mutants is a less pronounced accumulation of antisense transcripts in the cytoplasm than in the nuclei of mutant ovaries (Figure 6). Northern blot analysis confirms accumulation of sense *HeT-A* and *TART* transcripts and antisense *TART* transcripts in ovaries of *spn-E*/*spn-E* flies (Supplementary Figure 4). Long antisense *HeT-A* transcripts are not detected in this experiment. *TART* antisense transcripts are more abundant than sense ones in the ovaries of *spn-E*/+ flies (Supplementary Figure 4). This observation corresponds well with the previously revealed ratio of sense to antisense *TART* transcripts in wild type flies (37). Thus, *HeT-A* and *TART* antisense transcripts are the targets of the RNAi-based machinery and accumulated predominantly in the nuclear compartment of rasiRNA pathway mutant ovaries.

Accumulation of telomeric element transcripts of both polarities in ovarian nuclei of RNAi mutants suggests that the RNA silencing machinery and short RNAs guiding its specificity should be present in the nuclei. The intracellular distribution of *HeT-A*-specific rasiRNAs of both polarities was investigated by northern analysis of RNA from ovarian cell fractions. Distributions of sense and antisense *HeT-A* rasiRNAs do not differ markedly; both of them are present in cytoplasmic and nuclear fractions (Supplementary Figure 5). Thus, our results indicate that RNAi-based regulation of retrotransposon expression may occur in the nuclear compartment through the assistance of the rasiRNAs.

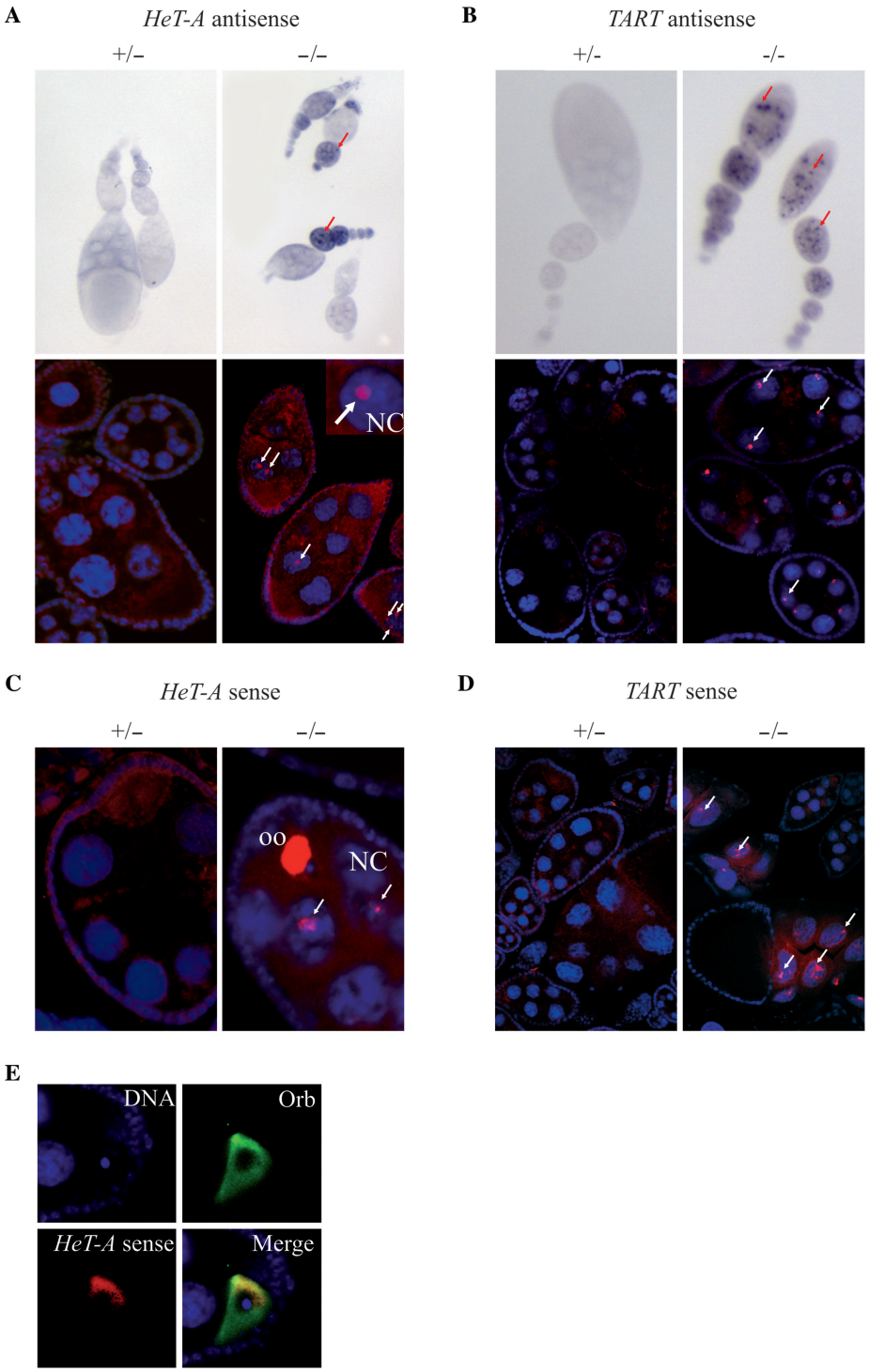

Figure 5. *HeT-A* and *TART* sense and antisense transcripts accumulate in *spn-E* mutants. The results were obtained by *in situ* RNA hybridization with *HeT-A* or *TART* riboprobes using anti-DIG AP-conjugated antibodies (**A** and **B**, upper panels) or anti-DIG rhodamin-conjugated antibodies (A and B, lower panels, **C**, **D** and **E**). Ovaries were dissected from *spn-E*^{1}/*TM3* (+/−) or *spn-E*^{1}/*spn-E*^{hls3987} (−/−) flies. DNA was stained with DAPI (blue). Oocyte and nurse cells are indicated (oo and NC, respectively). The arrows show the accumulation of transcripts in the nuclei of the nurse cells. *HeT-A* (A) and *TART* (B) antisense transcripts accumulate in the nuclei of the nurse cells in *spn-E* mutants. The insert (A) shows *HeT-A* antisense transcripts in the nurse cell nucleus. (C) *HeT-A* sense transcripts accumulate in the oocyte and in the nuclei of nurse cells in *spn-E* mutants. (D) *TART* sense transcripts accumulate in the cytoplasm and in the nuclei of nurse cells at late stages of oogenesis in *spn-E* mutants. (E) *HeT-A* sense transcripts (red) are localized in the cytoplasm of the oocyte in the *spn-E*^{1}/*spn-E*^{hls3987} flies where the Orb protein (green) is detected.

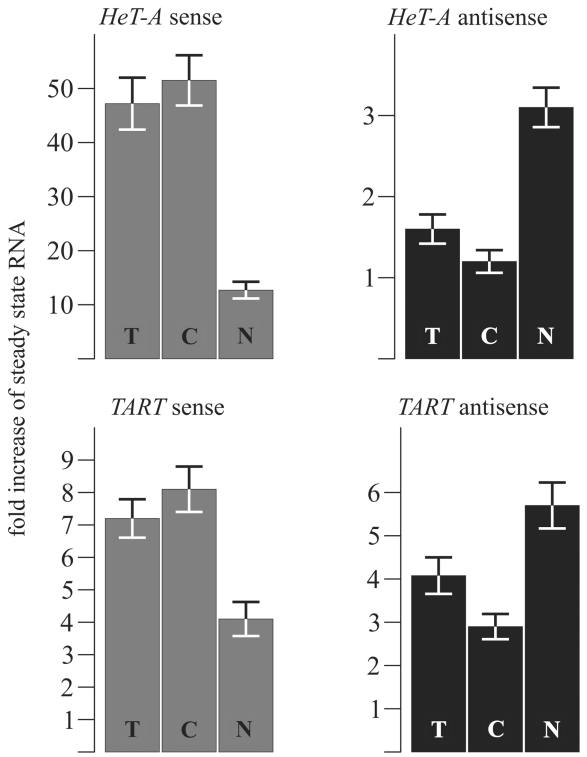

Figure 6. Cellular distribution of sense and antisense *HeT-A* and *TART* transcripts in the ovaries of *spn-E* mutants. RT-PCR analysis with strand-specific primers was done to compare *HeT-A* and *TART* sense and antisense transcript amounts in the total ovarian RNA (T), and in cytoplasmic (C) and nuclear (N) fractions from *spn-E* ovaries. The bars of histograms represent a ratio of *HeT-A(TART)* to *rp49* transcript abundance in the ovaries of transheterozygous *spn-E*^{1}/*spn-E*^{hls3987} flies related to this ratio in *spn-E*^{1}/*TM3* heterozygous females. Accumulation of both sense and antisense transcripts of telomeric retrotransposons is observed in the ovarian nuclei of mutant flies. In contrast to antisense transcripts, sense ones accumulate predominantly in cytoplasm.

DISCUSSION

In this study, we have investigated the peculiarities of the antisense expression of the telomeric retrotransposons in the *Drosophila* germline. We have demonstrated the antisense expression of non-LTR telomeric retrotransposon *HeT-A* from the internal promoter. Taking into account previously reported antisense expression of other retroelements, including telomeric *Drosophila* element *TART* (38), *Drosophila F*-element (24) and human L1 (21), it becomes clear that bidirectional transcription, at least of non-LTR retrotransposons, is an intrinsic feature of the retrotransposon expression. Sense and antisense promoters of the investigated retroelements are located in close proximity to each other. In this situation, it looks as if the enhancer-like element drives bidirectional expression of these elements. Enhancer-like elements, capable of functioning in either orientation, were identified in promoter regions of the *Drosophila F*-element and human L1 (53,54). For *TART* and *HeT-A* retrotransposons, a presence of such elements remains to be determined. Despite of the proximity of the sense and antisense *TART* promoters, they are activated at different stages of oogenesis (Figure 5B, D). Sense *TART* transcripts are detected only at late stages of oogenesis, while antisense transcripts begin to appear at the earlier stages. Antisense *Su*(*Ste*) transcripts involved in the RNAi-mediated silencing of testis-expressed *Stellate* genes were shown to accumulate in the nuclei of early spermatocytes before the appearance of sense transcripts (55). Thus, sense and antisense transcripts are coordinately expressed. Considering the earlier appearance of antisense transcripts, it is tempting to speculate that they might also play a role in short antisense RNA generation prior to sense transcript accumulation; this may occur with the assistance of the 'ping-pong' mechanism, guided by the maternally submitted sense rasiRNAs (12,56,57).

Another enigmatic feature of the antisense expression of retrotransposons is the presence of introns in antisense transcripts. Sense transcripts that serve as a template for transposition are usually not spliced, although such examples have been described (58–60). Antisense transcripts are spliced in alternative ways, as has been demonstrated for the *Drosophila TART* (38) and *HeT-A* (present study) elements and human L1 (21,23). The role of this splicing for retrotransposon silencing, if any, remains elusive.

5′ RACE analysis of endogenous *HeT-A* antisense transcripts revealed transcripts of a telomeric nature: specifically, read-through transcripts from the fourth chromosome. However, we were not able to identify some of the 5′ RACE sequences with the particular *HeT-A* copies despite the fact that the *D. melanogaster* stock with the sequenced genome was used in our study. *HeT-A*-related sequences were previously reported to be present in the pericentromeric regions of the *Drosophila* chromosomes (61). We do not exclude the possibility of a heterochromatic origin of the portion of antisense *HeT-A* transcripts.

We have shown for the first time that endogenous antisense transcripts of transposable elements are the targets of the RNAi machinery. Thus, sense expression and antisense expression, being the source of short RNAs of corresponding polarities, are mutually controlled via an RNAi-based mechanism. An interesting feature of the antisense transcript silencing is nuclear compartmentalization of the process. Vast amounts of natural antisense transcripts identified in the mouse transcriptome are present in the nucleus (62). Nuclear localization of antisense testes-expressed *Su*(*Ste*) repeat transcripts was previously reported (55). Northern analysis detected *TART* antisense transcripts in the nuclear fraction of the *Drosophila* cells (37). We have demonstrated the accumulation of the antisense retrotransposon transcripts in the nucleus as a result of rasiRNA pathway disruption. This suggests that an important pathway of the rasiRNA mechanism operates in the nucleus and participates in the biogenesis of antisense RNAs and possibly in the generation of a primary pool of rasiRNAs. Surprisingly, we did not observe significant accumulation of antisense RNAs in the cytoplasm, where they serve as a substrate for the 'ping-pong' amplification loop. The presence of multiple introns in antisense transcripts suggests a role of processing for the stabilization and nuclear-to-cytoplasm transport of these transcripts. Most likely, these transcripts are the targets of the nonsense-codon mediated decay (NMD)

machinery in the cytoplasm (63) because the antisense *HeT-A* and *TART* transcripts are noncoding. We believe that the regulation of the antisense expression may be a result of cumulative effects of both NMD and rasiRNA pathways. The role of the NMD machinery in the antisense retrotransposon transcript abundance remains to be determined.

RNAi-based machinery operates in the nucleus to induce transcriptional silencing through DNA or histone modification in plants, mammals and yeast (64). Evidence of the nuclear RNAi-mediated degradation of the target in human cells was previously reported (65). In *Drosophila*, the rasiRNA pathway component *spn-E* causes changes in the chromatin state of retroelements in ovaries (66). We have demonstrated that *HeT-A* and *TART* transcripts of both polarities accumulate in the germ cell nuclei as a result of rasiRNA pathway mutations. This fact clearly indicates that this mechanism controls transcript abundance in the nucleus. *HeT-A* and especially *TART* transcript hybridization signals in nuclei look like distinct bright dots, which may reflect the accumulation of nascent transcripts at the sites of transcription. This observation argues in favor of a transcriptional or co-transcriptional mechanism of regulation. The revealed nuclear population of the rasiRNAs may be involved in the rasiRNA-mediated silencing in the nucleus. The ratio between the amount of sense and antisense *HeT-A*-specific RNAs found in the nucleus and cytoplasm, however, appears to be the same. Most components of the *Drosophila* rasiRNA pathway were shown to be localized in the perinuclear cytoplasmic granules known as nuage (67–70), except for the nuclear PIWI protein (71). Thus, a distinct nuclear rasiRNA pathway may not exist; rather, a cross-talk between the cytoplasmic and nuclear rasiRNA pathway components ensures retrotransposon silencing in cell.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank V. Gvozdev for critical reading of this article, I. Olovnikov for helpful discussion, Y. Abramov for the assistance with fly maintenance and crosses and S. Lavrov, L. Olenina and A. Stolyarenko for methodological advices.

FUNDING

RAS program for Molecular and Cell Biology (to A.K.); Russian Foundation for Basic Researches (06-04-48493); Russian Foundation for Science School (3464.2008.4). Funding for open access charge: Russian Foundation for Basic Researches (06-04-48493).

Conflict of interest statement. None declared.

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