Telomere elongation is under the control of the RNAi-based mechanism in the *Drosophila* germline

Mikhail Savitsky,^{1,4} Dmitry Kwon,^{2,3} Pavel Georgiev,¹ Alla Kalmykova,^{2,5} and Vladimir Gvozdev^{2,6}

¹Department of the Control of Genetic Processes, Institute of Gene Biology, Russian Academy of Sciences, Moscow 119334, Russia; ²Department of Molecular Genetics of Cell, Institute of Molecular Genetics, Russian Academy of Sciences, Moscow 123182, Russia; ³Department of Molecular Biology, Moscow State University, Moscow 119899, Russia; ⁴Centre for Medical Studies of Oslo University, Moscow 199334, Russia

Telomeres in *Drosophila* are maintained by transposition of specialized telomeric retroelements *HeT-A*, *TAHRE*, and *TART* instead of the short DNA repeats generated by telomerase in other eukaryotes. Here we implicate the RNA interference machinery in the control of *Drosophila* telomere length in ovaries. The abundance of telomeric retroelement transcripts is up-regulated owing to mutations in the *spn-E* and *aub* genes, encoding a putative RNA helicase and protein of the Argonaute family, respectively, which are related to the RNA interference (RNAi) machinery. These mutations cause an increase in the frequency of telomeric element retrotransposition to a broken chromosome end. *spn-E* mutations eliminate *HeT-A* and *TART* short RNAs in ovaries, suggesting an RNAi-based mechanism in the control of telomere maintenance in the *Drosophila* germline. Enhanced frequency of *TART*, but not *HeT-A*, attachments in individuals carrying one dose of mutant *spn-E* or *aub* alleles suggests that *TART* is a primary target of the RNAi machinery. At the same time, we detected enhanced *HeT-A* attachments to broken chromosome ends in oocytes from homozygous *spn-E* mutants. Double-stranded RNA (dsRNA)-mediated control of telomeric retroelement transposition may occur at premeiotic stages, resulting in the maintenance of appropriate telomere length in gamete precursors.

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The problems of end-under-replication and stability of linear chromosomes are resolved by telomeres. The lengthening of terminal regions of linear eukaryotic chromosomes is often provided by RNA-templated addition of repeated DNA by reverse transcriptase enzyme, telomerase (Bryan and Cech 1999; Chan and Blackburn 2002). In most eukaryotes, telomeric DNA is maintained by the action of telomerase, which is responsible for the synthesis of short 6–8-nucleotide (nt) arrays using an RNA component as a template (Greider and Blackburn 1985). In contrast, telomeres of *Drosophila* are maintained as a result of retrotranspositions of specialized telomeric non-long-terminal repeat (LTR) *HeT-A*, *TAHRE*, and *TART* retrotranspositions (Biessmann et al. 1992b; Levis et al. 1993; for review, see Pardue and DeBaryshe 2003; Abad et al. 2004b). Retrotransposons are also found

in telomeric regions of such diverse organisms as *Bombix mori* (Okazaki et al. 1995; Takahashi et al. 1997), *Chlorella* (Higashiyama et al. 1997) and *Giardia lamblia* (Arkhipova and Morrison 2001). *HeT-A*, *TAHRE*, and *TART* are found at *Drosophila* telomeres in tandem arrays. *HeT-A*, the most abundant *Drosophila* telomeric element, contains a single ORF encoding a Gag-like RNA-binding protein, but lacks reverse transcriptase (RT). It is proposed that the RT necessary for its transposition might be provided in *trans*, perhaps by *TART* (Rashkova et al. 2002). *TART* ORF2 encodes a reverse transcriptase related to the catalytic subunit of telomerase (Levis et al. 1993). The recently discovered *TAHRE* element shows extensive similarity to *HeT-A*, but contains a second ORF, which encodes a reverse transcriptase (Abad et al. 2004b). A *HeT-A* promoter located in the 3' region of the element directs synthesis of a downstream neighbor (Danilevskaya et al. 1997). The *TART* element was shown to be transcribed bidirectionally using a putative internal sense promoter and antisense one that was localized within the 1-kb region of the *TART* 3' end (Danilevskaya et al. 1999). Maintenance of *Dro-*

Corresponding authors.

⁵E-MAIL allakalm@img.ras.ru; FAX 7-095-1960221.

⁶E-MAIL gvozdev@img.ras.ru; FAX 7-095-1960221.

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sophila telomere length is mediated by *HeT-A* and *TART* transpositions to chromosome ends as well as by terminal recombination/gene conversion (Mikhailovsky et al. 1999; Kahn et al. 2000). Most of the observed spontaneous attachments to telomeres are *HeT-A* transpositions (Biessmann et al. 1992a; Kahn et al. 2000; Golubovsky et al. 2001), but *TART* attachments were also detected (Sheen and Levis 1994).

The *spn-E* and *aub* genes, encoding an RNA helicase and a protein of Argonaute family, respectively (Gillespie and Berg 1995; Harris and Macdonald 2001; Kennerdell et al. 2002), were shown to be involved in double-stranded RNA (dsRNA)-triggered RNA interference (RNAi) in embryos (Kennerdell et al. 2002), in transcriptional silencing of transgenes (Pal-Bhadra et al. 2004), and in the control of *Drosophila* retrotransposon transcript abundance in the germline, especially in ovaries (Aravin et al. 2001; Kogan et al. 2003; Vagin et al. 2004). No effects of RNAi gene mutations on *HeT-A* and *TART* expression and telomere structure were observed in somatic tissues (Perrini et al. 2004). Here, we show that increased *HeT-A* and *TART* transcript abundance in ovaries, owing to RNAi mutations, is correlated with a high frequency of telomeric element attachments to broken chromosome ends. Addition of *HeT-A* or *TART* to a truncated X chromosome, with a break in the upstream regulatory region of *yellow*, activates *yellow* expression in aristae, which enables us to monitor the elongation events (Kahn et al. 2000; Savitsky et al. 2002). Using this genetic system, we studied the effects of RNAi mutations on the frequency and molecular nature of telomeric attachments. A high frequency of *TART* but not *HeT-A* attachments in heterozygous RNAi mutants suggests that *TART* may be the primary target of the RNAi-based silencing mecha-

nism. These results highlight for the first time the importance of *TART*, but not the more abundant *HeT-A* element, in *Drosophila* telomere maintenance. We found the disappearance of short *TART* and *HeT-A* RNAs in *spn-E* mutant ovaries, strongly suggesting an RNAi-based pathway in the control of telomere maintenance in the *Drosophila* germline.

Results

Different patterns of HeT-A and TART expression in ovaries of spn-E and aub mutants

HeT-A transcripts were shown earlier to accumulate in nurse cells and in the growing oocyte in the ovaries of *spn-E*¹ and *aub*^{QC42} mutants (Vagin et al. 2004). In situ RNA hybridization demonstrates the increased *TART* sense transcripts in cytoplasm of nurse cells, particularly at stage 10 of oogenesis in *spn-E*¹/*spn-E*³⁹⁸⁷ and *aub*^{QC42}/*aub*^{HN}, *aub*^{QC42}/*aub*^{N11}, *aub*^{QC42}/*aub*^{sting-1}, *aub*^{QC42}/*aub*^{ΔP-3a} heteroallelic flies (Fig. 1A). Ovaries of *spn-E* and *aub* homozygous mutants contain a small percentage of late-stage egg chambers as a result of strong disturbance of oogenesis (Gillespie and Berg 1995; Wilson et al. 1996). The late stages missing in the mutants led us to the incorrect conclusion that these RNAi mutations exert no effect on *TART* expression in ovaries (Vagin et al. 2004). The increase of *HeT-A* transcripts in the nurse cells and oocytes of *aub*^{QC42}/*aub*^{QC42}, *aub*^{QC42}/*aub*^{N11} and *aub*^{QC42}/*aub*^{ΔP-3a} individuals occurred in no more than a half of the examined ovaries (Fig. 1B) and sometimes resembled a mosaic *HeT-A* overexpression in separate ovarioles of the egg chamber. The *aub*^{QC42}/*aub*^{sting-1} trans-heterozygotes show no effect on *HeT-A* transcript

Figure 1. Effect of *spn-E* and *aub* mutations on the abundance of *HeT-A* and *TART* transcripts in ovaries observed by in situ RNA hybridization. (A) *TART* riboprobes detecting sense (s, upper panels) and antisense (as, lower panels) transcripts. *TART* sense transcripts are detected in the cytoplasm of nurse cells at the late stage of oogenesis in *spn-E*¹/*spn-E*^{his3987} and *aub*^{QC42}/*aub*^{ΔP-3a} ovaries. Identical staining was observed in *aub*^{QC42}/*aub*^{HN}, *aub*^{QC42}/*aub*^{N11}, and *aub*^{QC42}/*aub*^{sting-1} ovaries (data not shown). *TART* antisense transcript abundance is not affected by *spn-E* and *aub* mutations. (B) Detection of *HeT-A* sense transcripts in *aub* ovaries. *HeT-A* transcripts are detected in the cytoplasm of nurse cells and in the growing oocyte (arrows) in *aub*^{QC42}/*aub*^{ΔP-3a} ovaries. The two panels on the right show the mosaic character of *HeT-A* expression detected only in separate ovarioles. Identical staining was observed in *aub*^{QC42}/*aub*^{HN} and *aub*^{QC42}/*aub*^{N11} (data not shown). The *aub*^{QC42}/*aub*^{sting-1} trans-heterozygotes show no effect on *HeT-A* transcript abundance (data not shown). (C) Detection of sense *HeT-A* transcripts in eye-antennal discs of *spn-E* larvae. No difference in *HeT-A* expression in the cells of the morphogenetic furrow of eye-antennal discs of *spn-E*¹/+ and *spn-E*¹/*spn-E*¹ larvae is observed. Genotypes are indicated at the top of panels.

abundance (data not shown), whereas *TART* expression is up-regulated in the nurse cells of the all tested heteroallelic *aub* mutant flies. In contrast to *HeT-A*, we observed no *TART* transcript accumulation in the oocytes of RNAi mutants. Antisense *TART* transcripts are detected in the cytoplasm of nurse cells, but no increase in their abundance was found in RNAi mutants (Fig. 1A). Expression of *TART* sense and antisense transcripts in the nurse cells suggests the possibility of *TART*-specific dsRNA formation. *HeT-A* antisense transcripts are not detected in ovaries of RNAi mutants by in situ RNA hybridization (data not shown) and by Northern analysis in adult flies (Danilevskaya et al. 1999). Thus, an increase of both *HeT-A* and *TART* expression in ovaries of the *spn-E* and *aub* mutants was found, while the patterns of their expression are different: *HeT-A* transcripts are detected both in oocyte and nurse cells, whereas *TART* transcripts are accumulated substantially in the nurse cells at late stages.

HeT-A was shown to be transcriptionally active in proliferating somatic tissues such as brain and imaginal discs (George and Pardue 2003; Walter and Biessmann 2004). We observed no difference in the expression level of *HeT-A* in the actively dividing cells of the second mitotic wave in eye-antennal discs of *spn-E*¹/+ and *spn-E*¹/*spn-E*¹ larvae (Fig. 1C). These data highlight a specific role for RNAi genes in the regulation of telomeric retrotransposon expression in the germline.

Increased frequency of telomere attachments in *spn-E* and *aub* mutants

To study the frequency of telomeric element attachments to chromosome ends in RNAi mutants, we used the truncated X chromosomes (designated *y*^{TD}) with a break in the *yellow* locus that had been used earlier to investigate the effect of HP1 (heterochromatic protein 1) and Ku proteins on telomere elongation (Savitsky et al. 2002; Melnikova et al. 2005). The break is located in the upstream regulatory region and results in the *y*²-like phenotype with yellow aristae. Addition of *HeT-A* or *TART* retroelements can be monitored by a yellow-to-black change in aristae pigmentation (Savitsky et al. 2002, 2003). The *y*^{TD} chromosome is lethal as a result of several vital genes deleted in the region distal to the *yellow* gene. The *y ac* chromosome used as a homolog in this test has a deletion of the *yellow* and *achaete* genes but not of vital genes, and thus allowed us to monitor changes of *yellow* expression on the *y*^{TD} chromosome. This system provides an opportunity to estimate the frequency of telomeric element retrotranspositions to the broken chromosome end (Fig. 2A). Moreover, deletion of the *yellow* locus in the *y ac* X chromosome a priori obviates the possibility of terminal elongation using the homologous template to perform gene conversion (Kahn et al. 2000; Savitsky et al. 2002).

The emergence of flies with pigmented aristae was monitored in two lines carrying different *y*^{TD} chromosomes and *spn-E* or *aub* mutations in a heterozygous state (see Materials and Methods). Two alleles of *spn-E*,

Figure 2. Molecular structure of the *HeT-A/TART* attachments to the broken chromosome end. (A) Yellow (left)-to-black (right) change in pigmentation of aristae (indicating by arrows) is a result of addition of *HeT-A* or *TART* retroelements to the broken X chromosome truncated upstream of the *yellow* gene. (B) Schematic representation of *HeT-A/TART* attachments to the broken *yellow* gene. The *yellow* region is shown as a yellow box. The horizontal red and green arrows show *HeT-A/TART* attachments, respectively. Vertical arrows point to *EcoRI* restriction sites. The solid horizontal line indicates a *yellow* probe used for Southern analysis. The junctions between *yellow* and new DNA attachments were amplified using primers schematically indicated as small horizontal arrows. (C) Southern blot analysis of DNAs prepared from the progeny of individual *y*^{TD}/*y ac*; *spn-E*¹/*MKRS* flies with yellow aristae (without attachments, lane 10) and black aristae (with *TART* or *HeT-A* attachments, lanes 1–6 and 7–9, respectively). DNA was digested by *EcoRI* enzyme. The filter was hybridized with the *yellow* probe indicated in B. The 4-kb fragment in lane 10 reflects the distance from the *EcoRI* site in *yellow* to the truncated end of the broken chromosome. The 5–15-kb bands in lanes 1–6 correspond to the different size attachments of *TART* elements. The 5.0-kb bands in lanes 7–10 correspond to attachments of *HeT-A* elements carrying a conservative *EcoRI* site in the 3' region. (D) Southern analysis of DNA isolated from F2 progeny of four females with black aristae (*TART* attachments), the descendants of an individual *y*^{TD}/*y ac*; *aub*^{QC42}/*CyO* fly with yellow aristae. Hybridized fragments are identical, confirming *TART* addition at the earlier premeiotic stages of oogenesis. DNA size markers are indicated in kilobases.

*spn-E*¹, and *spn-E*^{hls3987}, as well as *aub*^{QC42} were used. Table 1 shows the combined results for both *y*^{TD} chromosomes, indicating an increase of the emergence of flies with black aristae. Homozygous *spn-E* and *aub* mutant females are sterile; thus, the observed effect may be

Table 1. Frequencies of terminal attachments in *spn-E* and *aub* mutants

RNAi mutations	No. of flies displaying a new y phenotype/total no. in generation					% Q ($F_2 - F_5$) ^a
	F ₁	F ₂	F ₃	F ₄	F ₅	
<i>spn-E</i> ¹ /MKRS	1/569	3/146	41/1281	13/1162	11/759	2.1
<i>spn-E</i> ^{hls3987} /MKRS	1/556	36/416	18/156	35/247	37/998	6.5
<i>aub</i> ^{QC42} /CyO	0/651	3/306	10/787	17/2629	—	0.8
Control ^b	0/735	0/1005	0/595	0/744	0/824	0.0

^aQ, average frequency of visible events signifying a new *yellow* phenotype(s) versus total number of flies scored (as a percentage).

^bCombined results of fly score obtained for *y*^{TD}/*y ac*; TM6/MKRS and *y*^{TD}/*y ac*; CyO/*If* lines.

attributed to the effect of a single dose of mutant allele. No attachments were detected in control lines. Both *spn-E* mutations have a strong dominant effect on the frequency of attachments resulting in a 100 times increase in the appearance of flies with black-colored aristae as compared with the earlier observed frequency, 0.04%, of spontaneous telomeric attachments to broken chromosome ends (Kahn et al. 2000). Examination of the *aub* mutant effect also revealed a considerable, but smaller increase in the frequency of putative terminal attachments. We suggest that both RNAi genes control telomere elongation in *Drosophila*.

Heterozygous *spn-E* and *aub* mutations increase TART attachment to broken chromosome ends

To detect the nature of the attached elements, the junctions between terminal *yellow* sequences and retrotransposon attachments were analyzed. Genomic DNAs were prepared from the progeny of randomly chosen individual females with restored aristal pigmentation. The junctions between the newly transposed mobile elements and the *yellow* DNA were determined by DNA amplification with two primers from the *yellow* gene and the most conserved regions in the 3' part of *HeT-A* or *TART* elements (Fig. 2B). Table 2 summarizes the result of PCR analysis. Surprisingly, attachments of *HeT-A* occurred significantly less frequently than those of *TART* elements in *spn-E*¹ mutants. No *HeT-A* additions were observed in the lines carrying *spn-E*^{hls3987} and *aub*^{QC42} mutations.

Sequencing of PCR fragments confirmed that, in most cases, the attached elements occurred at different positions of *yellow*, which is shortened (70–75 base pairs [bp] per generation) as a result of the expected terminal *yellow* DNA loss (Biessmann and Mason 1988; Levis et al. 1993; Supplementary Fig. 1). The sequences of *HeT-A* 3' termini (5'-CCAGCAAAGTTA-3') were conserved and terminated by short oligo(A)_{3–11} stretches. *TART* attachments are characterized by stretches of oligo(A)_{20–28} at their 3' termini. Most of the *TART* elements attached to the broken chromosome are attributed to class A, B, or C1 (GenBank sequences U02279, U14101, and AY600955, respectively). The presence of oligo(A) tails, conserved 3' sequences of attached elements and attachments to different sites of the shortened *yellow* gene, confirm the retrotransposition mechanism for *TART* and *HeT-A* attachments to a broken chromosome end.

Genetic screening of terminal elongation events in the progeny of individual *y*^{TD}/*y ac*; *spn-E*^{hls3987}/MKRS and *y*^{TD}/*y ac*; *aub*^{QC42}/CyO females revealed three cases in which several flies with black aristae arose from the same cross. PCR analysis revealed *TART* attachments in all cases. Sequencing in each of these cases revealed that clusters of flies with black aristae contained identical junctions (Supplementary Fig. 1). Hence, terminal elongation may occur at premeiotic stages of oogenesis.

Southern analysis confirmed elongation of a terminally deleted chromosome (Fig. 2C). Genomic DNA from flies with yellow aristae, A[−] (in other words, without *HeT-A*/*TART* attachments), and black aristae, A⁺ (with attachments), were digested with EcoRI. A PCR fragment from *yellow* upstream of the EcoRI site was used as a probe (Fig. 2B). EcoRI recognizes a site in the *yellow* sequence ~4 kb proximal to the terminus of the original broken chromosome, producing a fragment corresponding in size to A[−] genomic DNA. EcoRI is a conserved site in the 3' region of most *HeT-A*s. No EcoRI recognition sites were detected in most *TART* sequences. Fragments of ~5.0 kb encompassing ~4 kb of terminal *yellow* sequence and ~1.0 kb of *HeT-A* are detected by Southern in A⁺ genomic DNA of the flies in which PCR analysis earlier revealed *HeT-A* attachments (Fig. 2C, lanes 7–10). Southern analysis reveals fragments of different sizes (from ~5 to 15 kb) when DNA from A⁺ flies with *TART* additions is digested (Fig. 2C, lanes 1–6). This indicates either attachment of *TART* elements having polymorphic EcoRI site or addition of truncated *TART* elements of different sizes, because generation of retroelement copies truncated at the 5' end is a characteristic feature of RT as a result of its low processivity. This latter observation is consistent

Table 2. Identification of telomeric elements attached to the terminally deleted X chromosome in *spn-E* and *aub* mutants

Attachment type	RNAi mutations		
	<i>spn-E</i> ¹	<i>spn-E</i> ^{hls3987}	<i>aub</i> ^{QC42}
<i>TART</i>	29	38	16
<i>HeT-A</i>	6	—	—
Total ^a	35	38	16

^aNumber of analyzed lines with black-colored aristae.

with a retrotransposition mechanism of *TART* attachments to a broken terminus.

Fragments detected in genomic DNA of flies from individual clusters, were of the same size (Fig. 2D), confirming *TART* addition at the earlier premeiotic stages of oogenesis.

Thus, a dominant effect of the tested RNAi mutations was observed in the increased frequency of *TART* retrotranspositions to broken chromosome ends. However, we failed to detect an increase in the number of total genomic *HeT-A* and *TART* copies in *spn-E*¹/+, *spn-E*^{hls3987}/+ and *aub*^{QC42}/+ lines (data not shown). This observation suggests that the telomere length of native chromosomes is not increased significantly. Telomere elongation was detected in *Su(var)205* mutants only in those lines that have been maintained for many years (Savitsky et al. 2002), indicating that attachment of retrotransposons to native telomeres is slower than that to a truncated chromosome or that the aristae test is more sensitive than tests for overall telomere length.

HeT-A transposes to chromosome ends in the oocytes of homozygous spn-E mutants

HeT-A expression was shown to be strongly up-regulated in the ovaries of homozygous *spn-E*¹ females, resulting in accumulation of *HeT-A* transcripts at the anterior of the oocyte (Vagin et al. 2004). Since we are unable to estimate frequency of terminal attachments in a progeny of sterile homozygous RNAi mutants, we studied a correlation between *HeT-A* transcript abundance and telomere elongation in ovaries of homozygous *spn-E* flies carrying a *y*^{TD} chromosome. Approximately 50 mature oocytes, which are rarely produced in ovaries of homozygous *spn-E* mutants, were dissected from 10 *spn-E*¹/*spn-E*¹ flies with yellow aristae, and DNA was isolated. This DNA sample contains oocyte DNA as well as DNA from surrounding follicular cells where no effect of RNAi mutations on *HeT-A* expression was observed. Genomic DNA was isolated also from carcasses of females after gonad removal. PCR analysis using primers from *yellow* and the 3' conserved termini of *HeT-A/TART*, followed by nested PCR was performed. A set of bands was detected in the DNA sample from oocytes when *HeT-A*-specific primers were used. No PCR bands were revealed in DNA from carcasses. Sequencing of PCR products revealed at least five independent *HeT-A* attachments to truncated chromosome termini (Supplementary Fig. 2A). The same result was obtained when oocytes from a heteroallelic *y*^{TD}/*y ac*; *spn-E*¹/*spn-E*^{hls3987} female were analyzed (Supplementary Fig. 2B). In this case, seven independent *HeT-A* attachments were observed in the DNA isolated from oocytes of an individual female. Thus, drastic accumulation of *HeT-A* transcripts in *spn-E*¹/*spn-E*¹ and *spn-E*¹/*spn-E*^{hls3987} oocytes results in a high frequency of *HeT-A* transpositions to the chromosome end, but no *TART* attachments were found in these cases.

RT-PCR analysis of TART and HeT-A expression in spn-E ovaries

To elucidate a phenomenon of preferential *TART* attachments in ovaries of heterozygous *spn-E* mutants, but much more frequent *HeT-A* attachments in ovaries of *spn-E* homozygous flies, we tested for a dosage effect of RNAi mutations on the expression of both retrotransposons. The levels of *HeT-A* and *TART* transcripts were compared by RT-PCR using an oligo(dT) primer in ovaries of *y*^{TD}/*y ac*; *TM6/MKRS*, *y*^{TD}/*y ac*; *spn-E*¹/*MKRS* and *y*^{TD}/*y ac*; *spn-E*¹/*spn-E*^{hls3987} flies. Since the level of antisense *TART* and *HeT-A* transcripts detected in situ RNA hybridization is not affected in RNAi mutants, observed changes may be attributed to the abundance of sense *TART* and *HeT-A* transcripts. We observed increased *TART* transcript abundance in ovaries from heterozygous *spn-E*¹ flies and, further, an increase of *TART* expression in *spn-E*¹/*spn-E*^{hls3987} ovaries as compared with *y*^{TD}/*y ac*; *TM6/MKRS* ovaries (Fig. 3A). However, while a statistically significant but small dosage effect of *spn-E*¹ on *HeT-A* expression was detected in ovaries of *spn-E*¹/+ females, a drastic accumulation of *HeT-A* transcripts was observed in ovaries of *spn-E*¹/*spn-E*^{hls3987} flies (Fig. 3B). Thus, strong derepression of *HeT-A* in homozygous *spn-E* mutants provides an explanation for the high frequency of *HeT-A* attachments to the broken chromosome ends in ovaries from homozygous mutant females. *TART* may be considered a primary target of RNAi genes owing to the obvious dosage effect of *spn-E* and *aub* (data not shown) mutations on its expression.

spn-E mutations eliminate short HeT-A and TART RNAs in ovaries

aub and *spn-E* are required for artificial RNAi in oocytes and embryos (Kennerdell et al. 2002). Mutations in both

Figure 3. Suppression effect of *spn-E* on *TART* and *HeT-A* expression. RT-PCR analysis of *TART* (A) and *HeT-A* (B) transcript amounts in ovaries of *y*^{TD}/*y ac*; *spn-E*¹/*MKRS* (*spn-E*/+) and *y*^{TD}/*y ac*; *spn-E*¹/*spn-E*^{hls3987} (*spn-E/spn-E*) flies. Histograms display the quantification of five experiments. Bars represent the ratios of *TART* or *HeT-A* to *rp49* transcript abundance in *y*^{TD}/*y ac*; *spn-E*¹/*MKRS* (*spn-E*/+) and *y*^{TD}/*y ac*; *spn-E*¹/*spn-E*^{hls3987} (*spn-E/spn-E*) ovaries related to this ratio in *y*^{TD}/*y ac*; *TM6/MKRS* (+/+). Dosage effect of *spn-E* on *TART* expression is demonstrated (A), while drastic accumulation of *HeT-A* transcripts is observed only in ovaries of *spn-E/spn-E* flies (B).

genes eliminate the short *Suppressor-of-Stellate* RNA involved in repression of homologous, testes-expressed *Stellate* genes by a natural dsRNAi-mediated mechanism (Aravin et al. 2001, 2004). *HeT-A*- and *TART*-specific short RNAs were observed among cloned short RNAs in *Drosophila* (Aravin et al. 2003). We tested for the presence of short RNA species homologous to *TART* and *HeT-A* in the ovary RNA isolated from *spn-E* mutants and the wild line *Gaiano*, which is characterized by long telomeric arrays of *HeT-A* and *TART* elements (Savitsky et al. 2002; Siriaco et al. 2002). Northern hybridization using *TART* and *HeT-A* probes to detect antisense transcripts revealed the presence of heterogeneous 26–29-nt *TART* and *HeT-A* RNA species in the ovarian RNA isolated from *Gaiano* and heterozygous *spn-E*/+ flies (Fig. 4A,B). The stronger signal for *Gaiano* is expected, because this line contains longer telomeric arrays. However, no *HeT-A* or *TART* short RNAs were detected in RNA from heteroallelic *spn-E*^{hls3987}/*spn-E* ovaries. Expression of microRNA (*mir-13b1*) in ovaries is not affected by *spn-E* mutation (Fig. 4A,B), corroborating earlier observations that *spn-E* does not affect miRNA production in the germline (Aravin et al. 2004). The signal corresponding to *TART* short RNA in *spn-E*¹/+ ovaries is faint (Fig. 4A). This observation is consistent with the increased frequency of *TART* attachments to the broken chromosome ends in *spn-E*¹/+ individuals. A mutation in the not yet identified *Tel* locus in the *Gaiano* strain may be responsible for abnormal telomere elongation in this stock (Siriaco et al. 2002). The presence of short *HeT-A* and *TART* RNAs in *Gaiano* indicates that this locus is not related to the production or stabilization

of telomeric short RNAs. Our results show a negative correlation between the expression and transposition of telomeric elements and the presence of short RNAs guiding dsRNA-mediated silencing.

Discussion

An RNAi-based mechanism was proposed to evolve in order to immobilize transposable elements and was found to control expression of endogenous transposable elements and their mobility in different species (Wu-Scharf et al. 2000; Aravin et al. 2001; Sijen and Plasterk 2003; Shi et al. 2004; Svoboda et al. 2004; Kalmykova et al. 2005). *Drosophila* telomeres are maintained by successive transpositions of specialized telomeric retroelements *HeT-A* and *TART*. In this study, we show that transposition of both telomeric elements is under the control of the *spn-E* and *aub* genes, known to be related to the RNAi machinery. Hence, an RNAi-based mechanism may be considered not only as a defense against retrotransposon expansion, but also as a regulatory system responsible for proper telomere length maintenance in *Drosophila*.

spn-E is required for appropriate localization of mRNA and proteins involved in the establishment of axis formation in the embryo and encodes a member of the DEAD/DE-H protein family possessing RNA-binding and RNA helicase activity (Gillespie and Berg 1995). *aub* encodes a protein of the Argonaute family that was shown to be a component of the RNAi effector complex RISC (Tomari et al. 2004). *aub* and *spn-E* mutations strongly diminished effects of the injected dsRNA into mature oocytes (Kennerdell et al. 2002). Both genes are implicated in small interfering RNA (siRNA)-dependent silencing of testis-expressed *Stellate* genes (Aravin et al. 2001). Thus, *spn-E* and *aub* are components of RNAi-based silencing pathways in *Drosophila*. Mutations in these genes result in the derepression of a wide spectrum of retrotransposons in the germline, including the *HeT-A* telomeric element (Aravin et al. 2001; Stapleton et al. 2001; Kogan et al. 2003; Vagin et al. 2004). Here, we demonstrate that *spn-E* and *aub* mutations increase the frequency of telomeric element retrotranspositions to broken chromosome termini, suggesting that the RNAi machinery controls telomere length in *Drosophila*.

Both telomeric elements are shown to be the targets of RNAi. The present results emphasize the differences in the response of *HeT-A* and *TART* elements to RNAi mutations. Surprisingly, two different *spn-E* mutant alleles and an *aub* mutation in the heterozygous state increase considerably *TART* mobility, whereas attachments of *HeT-A* to broken chromosome ends were detected much more rarely in *spn-E*¹/+ ovaries and are not observed in ovaries of *spn-E*^{hls3987}/+ and *aub*^{QC42}/+ flies. One copy of a *spn-E* mutation is sufficient to increase *TART* transcript abundance. Strong accumulation of *HeT-A* transcripts is found only in homozygous mutants, correlating with a high frequency of *HeT-A* attachments to the broken chromosome ends in the developing oocytes.

Figure 4. Effect of *spn-E* mutation on the presence of the short *HeT-A* and *TART* RNAs in ovaries. Northern analysis of RNA, isolated from ovaries of *Gaiano* (+/+), *spn-E*¹/+ (+/-), and *spn-E*¹/*spn-E*^{hls3987} (-/-) flies. (A) Hybridization with the *TART* sense riboprobe reveals short *TART* RNAs of 27–29 nt in size in *Gaiano*, a faint signal in heterozygous ovaries, and no signal in ovaries of heteroallelic *spn-E* flies. (B) Hybridization with the *HeT-A* sense riboprobe. Short *HeT-A* RNAs are absent in heteroallelic *spn-E*¹/*spn-E*^{hls3987} (-/-) flies. The lower panels represent hybridization with an oligonucleotide complementary to the *mir-13b1* microRNA. ³³P-labeled RNA oligonucleotides were used as size markers.

This observation argues that *TART* is a primary target of the RNAi machinery in ovaries.

TART and *HeT-A*, in spite of sharing the region of integration, are dissimilar in their structure and expression strategy (Danilevskaya et al. 1999). While both sense and antisense *TART* transcription was shown, antisense transcripts were more abundant (Danilevskaya et al. 1999). In situ RNA analysis detected sense and antisense *TART* transcripts in the cytoplasm of nurse cells in the late-stage egg chambers, suggesting a possibility of dsRNA formation. However, we found that the level of antisense *TART* transcripts is not affected in RNAi mutants. Only sense *HeT-A* transcription was observed by Northern (Danilevskaya et al. 1999) or by in situ RNA analyses (Walter and Biessmann 2004; our data). Nevertheless, *HeT-A*- and *TART*-specific siRNAs were revealed among the cloned short RNA species in *Drosophila* (Aravin et al. 2003), and we detected short RNAs corresponding to both *HeT-A* and *TART* elements by Northern analysis. Antisense *HeT-A* RNA is probably transcribed at a low level from an unidentified promoter, possibly, from the *HeT-A* internal region. Actually, a low level of antisense activity of the *HeT-A* 3' end was observed (M.-L. Pardue, pers. comm.). While *TART* transcripts were observed only in the nurse cells, *HeT-A* transcripts were detected both in the growing oocyte and nurse cells. We propose that *TART* is a primary target of the RNAi controlling system, since one dose of an RNAi mutation causes preferential *TART*, but not *HeT-A*, attachments to broken chromosome ends in ovaries. On the contrary, one dose of a mutant *Su(var)205* gene considerably increased the frequency of *HeT-A* rather than *TART* attachments to the chromosome ends (Savitsky et al. 2002). Thus, we observed a specific effect of RNAi components on telomeric element expression. Although *TART* copies are much less abundant in the genome than *HeT-A* and no *TART* elements are detected in some telomeres (Levis et al. 1993; Abad et al. 2004a), *TART* is a conserved component of telomeres in distant *Drosophila* species (Casacuberta and Pardue 2002, 2003). *TART* was considered as a source of RT production, thus ensuring retrotranspositions of both *TART* and *HeT-A* elements (Rashkova et al. 2002). One may propose that *TART* supplies an RNAi-regulated template for RT production, thus providing telomere-specific transpositions of both elements.

Drosophila telomeres contain a multisubunit protein complex forming a chromosome cap protecting chromosomes from DNA repair and end-to-end fusions (Cenci et al. 2005). However, no *HeT-A* or *TART* sequences were detected at the stably maintained broken chromosome end that is protected from telomere fusions (Biessmann et al. 1990; Mason and Biessmann 1995). Thus, a sequence-independent system performs telomere capping functions. The capping complex contains HP1 (Fanti et al. 1998), HOAP (HP1/ORC associated protein) (Cenci et al. 2003), as well as ATM-kinase and DNA repair MRN complex and the Ku70/Ku80 heterodimer (Bi et al. 2004; Ciapponi et al. 2004; Oikemus et al. 2004; Silva et al. 2004; Song et al. 2004; Melnikova et al. 2005). HP1 and

the Ku heterodimer act also as negative regulators of telomere elongation by retrotransposition of telomeric elements (Savitsky et al. 2002; Melnikova et al. 2005). Deficiencies that remove either the *Ku70* or the *Ku80* gene increase the transposition rate of *HeT-A* and *TART* elements but exert no effect on the *HeT-A* expression, suggesting that Ku proteins control the accessibility of the telomere to transposition events (Melnikova et al. 2005). At the same time, mutations in the *Su(var)205* gene increase both transcript abundance of *HeT-A* and *TART* and the frequency of their attachments to chromosome ends (Savitsky et al. 2002; Perrini et al. 2004). RNAi affects both telomeric retrotransposon expression and the rate of transposition to the telomere. Probably, this effect is mediated through HP1 recruitment and silencing of *HeT-A* and/or *TART* chromatin.

siRNAs produced from telomeric elements *TART* and *HeT-A* belong to the long size class (25–29 nt) in contrast to 21–22-nt RNAs guiding post-transcriptional RNAi (Elbashir et al. 2001). In plants, long siRNAs are associated with RNA-directed DNA methylation and play an essential role in the transcriptional retrotransposon silencing (Hamilton et al. 2002). dsRNA and proteins of the RNAi machinery can direct chromatin alteration to homologous DNA sequences and induce transcriptional silencing (Matzke and Birchler 2005). RNAi mutations cause delocalization of HP1 in yeast and *Drosophila* (Hall et al. 2002; Volpe et al. 2002; Pal-Bhadra et al. 2004). Actually, the increase in accessibility of *HeT-A* chromatin and its enrichment in K9-acetylated H3 histone were revealed in ovaries of *spn-E* mutants (M. Klenov and S. Lavrov, unpubl.). It is also possible that *TART* and/or *HeT-A* short RNAs can be targeted to telomeric repeats in a transcriptional silencing complex.

RNAi disruption affects neither *HeT-A* and *TART* expression, nor telomere fusions in somatic cells (Perrini et al. 2004). We failed to observe the effect of *spn-E* mutations on *HeT-A* expression, even in actively dividing cells of imaginal discs, where *HeT-A* expression was found (George and Pardue 2003; Walter and Biessmann 2004). Our data indicate a crucial role of the RNAi machinery in the regulation of telomere elongation in germinal cells. The appearance of a cluster of individuals with identical retroelement attachments indicates that dsRNA-mediated control of terminal elongation may occur at premeiotic stages of oogenesis.

We have demonstrated for the first time that expression and retrotransposition of specific telomeric repeats is under control of an RNAi-based system in the *Drosophila* germline. In this case, the telomerase-dependent mechanism of telomere stability is substituted by retrotranspositions. Interestingly, telomerase-dependent telomere functioning during meiosis in the yeasts *Schizosaccharomyces pombe* and *Tetrahymena* is also under the control of RNAi machinery (Hall et al. 2003; Mochizuki and Gorovsky 2005). These observations and our data indicate that dsRNA-mediated regulation of telomere dynamics in the germline may be a general phenomenon independent of a mode of telomere maintenance.

Materials and methods

Drosophila strains and genetic crosses

The strains bearing *spindle-E* (*spn-E*) mutations were *ru¹ st¹ spn-E¹ e¹ ca¹/TM3*, *Sb¹ e^s* (bearing a point mutation in the helicase domain), and *ru¹ st¹ spn-E^{hls3987} e¹ ca¹/TM3*, *Sb¹ e^s* (caused by *P*-element insertion). The *aub* mutants were *aub^{QC42}/CyO*, *aub^{HN}/CyO*, *aub^{N11}/CyO*, *aub^{sting1}/CyO*, and *aub^{ΔP3a}/CyO*.

To study the frequency of *HeT-A/TART* attachments to broken chromosome ends, we used a terminally truncated chromosome with a break in the *yellow* locus designated *y^{TD}*. The *y^{TD}* chromosome is lethal. The homologous *y ac X* chromosome has a deletion of *yellow* and *achaete* loci but not of any vital genes. Two lines, *y^{TD4}/y ac* and *y^{TD5}/y ac*, carrying deficiencies terminating in the region ~1.3–1.5 kb upstream of the *yellow* transcription start site, were used. The *y^{TD}/y ac*; *TM6/MKRS* females were crossed to *y ac*; *spn-E¹/MKRS* males. In the progeny, *y^{TD}/y ac*; *spn-E¹/MKRS* females were crossed to *y ac*; *spn-E¹/MKRS* males for five successive generations to determine the appearance of flies with black aristae. The same crossing was used to study the *spn-E^{hls3987}* allele. The *y^{TD}/y ac*; *TM6/MKRS* line was used as a control. *y^{TD}/y ac*; *CyO/If* females were crossed to *y ac*; *aub^{QC42}/CyO* males to obtain *y^{TD}/y ac*; *aub^{QC42}/CyO* females. The *y^{TD}/y ac*; *CyO/If* line was used as a control.

Northern and in situ RNA analyses

Northern analysis of short RNAs was done according to Aravin et al. (2001). ³²P-labeled riboprobes corresponding to the sense strands of *TART* and *HeT-A* were synthesized. In situ RNA analysis was carried out according to the earlier described procedure (Kogan et al. 2003) using DIG-labeled strand-specific *TART* and *HeT-A* riboprobes. Plasmids containing the cloned PCR fragments of retrotransposons were used as transcription templates. *TART*: A PCR-amplified fragment using primers 5'-TTCTATCAACAGGCTGTCCACAGGTT-3' and 5'-CCTTCG TAGTCGGGTAGGATTATTCGT-3' corresponding to GenBank sequence DMU02279 was cloned into pBS SK[−]. *HeT-A*: A PCR-amplified fragment using primers 5'-TCATTGACGATAC CACGCCATC-3' and 5'-TCCGGGTGCGTTTATAGGTGAG-3' corresponding to GenBank sequence DMU06920 was cloned into pBS SK[−]. Hybridization with ³²P-end-labeled oligonucleotide 5'-ACTCGTCAAAATGGCTGTGATA-3' complementary to *mir13b1* was used as a loading control.

PCR and RT-PCR analyses

DNA samples were isolated from the F2 progeny of individual *y^{TD}/y ac*; *spn-E¹/MKRS*, *y^{TD}/y ac*; *spn-E^{hls3987}/MKRS* or *y^{TD}/y ac*; *aub^{QC42}/CyO* females with black aristae according to the standard method (Ashburner 1989). PCR was done using different combinations of primers corresponding to *TART/HeT-A* and *yellow* sequences as follows: *TART*-specific primers: *TART1*, 5'-CGAAACGCAACAACAAAATGG-3'; *TART2*, 5'-AAGGA TGGGCAACAGTGCTATC-3'; *TART3*, 5'-TGGACGGCCAG AACGTGAACCA-3'; *HeT-A*-specific primers: *A2H*, 5'-TTGC AATATGTTAATGTTACCAGTCCATG-3'; *M1*, 5'-CTGTCT CCGTACCTCCACCAGC-3'; *M3*, 5'-CCTATATATTGCACA TACTGTAATCAAAG-3'; *yellow*-specific primers corresponding to GenBank sequences X06481 and X04427: *y13*, 5'-AAT ATTTTGTTCGCTAGTTATTG-3'; *y12*, 5'-ATTGGATTT CGATTGGGCGTCAC-3'; *y5*, 5'-CAGGAGGCTCGTGCATA GAATGC-3'; *y9*, 5'-GGTTCAGTGTTCCGGTAATCAGGTG-3';

y17, 5'-AAGACGGCGTCACCAAGGGTATC-3'; *7y*, 5'-CTTG CCGCGATGGTCATTAGAGC-3'. PCR fragments were sequenced directly or cloned in pTR19R.

To reveal *TART/HeT-A* attachments in oocytes of sterile *spn-E* mutants, genomic DNA was isolated from oocytes of *y^{TD}/y ac*; *spn-E¹/spn-E¹* and *y^{TD}/y ac*; *spn-E¹/spn-E^{hls3987}* females with yellow-colored aristae and carcasses of flies after gonad removal. PCR with primers *y12* or *y17* and *A2H/TART1* following by nested PCR with *y13* or *y9* and *M1/TART2* was performed using *y^{TD}/y ac*; *spn-E¹/spn-E¹* or *y^{TD}/y ac*; *spn-E¹/spn-E^{hls3987}* DNAs, respectively. PCR products were cloned and sequenced.

RT-PCR was done according to described procedure (Aravin et al. 2001) using pairs of primers corresponding to *HeT-A* and *TART* as indicated in the previous section of Materials and Methods, and with *rp49* (5'-ATGACCATCCGCCACGAT AC-3' and 5'-CTGCATGAGCAGGACCTCCAG-3') as a loading control. cDNA was synthesized using an oligo(dT) primer. The results of RT-PCR analyses were evaluated using the program BioDocAnalyze1.0.

Southern analysis was performed according to the standard method (Ashburner 1989) using as a probe a ³²P-labeled fragment of *yellow* obtained by primers *7y* and *y9*.

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