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

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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.

**Keywords**: Telomere; RNAi; retrotransposon; *HeT-A*; *TART*; germline

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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 *Bombyx 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].
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. 2000], and in the control of Drosophila retrotransposon transcript abundance in the germ line, 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 mechanism. 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 germ line.

**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 and aub transcripts [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/spn-E<sup>spn-E<sup>9887</sup>** and aub<sup>GC42</sup>/aub<sup>H17N</sup>, aub<sup>GC42</sup>/aub<sup>N11</sup>, aub<sup>GC42</sup>/aub<sup>string-1</sup>, aub<sup>GC42</sup>/aub<sup>AP-3a</sup>**/H9004 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<sup>GC42</sup>/aub<sup>GC42</sup>, aub<sup>B3</sup>/aub<sup>N11</sup> and aub<sup>GC42</sup>/aub<sup>AP-3a</sup>** 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<sup>GC42</sup>/aub<sup>string-1</sup> trans-heterozygotes show no effect on HeT-A transcript abundance 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/<sup>spn-E<sup>B33017</sup>** and aub<sup>GC42</sup>/aub<sup>AP-3a</sup>** ovaries. Identical staining was observed in aub<sup>GC42</sup>/aub<sup>H17N</sup>, aub<sup>GC42</sup>/aub<sup>N11</sup> and aub<sup>GC42</sup>/aub<sup>string-1</sup> 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<sup>GC42</sup>/aub<sup>AP-3a</sup>** 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<sup>GC42</sup>/aub<sup>H17N</sup> and aub<sup>GC42</sup>/aub<sup>N11</sup> [data not shown]. The aub<sup>GC42</sup>/aub<sup>string-1</sup> 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<sup>1</sup>+/+ and spn-E<sup>1</sup>/spn-E<sup>1</sup> 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 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 oocytes 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 oocytes 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/+ and spn-E∥/spn-E∥ 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 yTD] 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 yTD-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 yTD 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 yTD 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 yTD chromosomes and spn-E or aub mutations in a heterozygous state [see Materials and Methods]. Two alleles of spn-E, spn-E∥, and spn-E∥hI39587, as well as aubQC42 were used. Table 1 shows the combined results for both yTD 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
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 \(\text{HeT}-A\) or TART elements [Fig. 2B]. Table 2 summarizes the result of PCR analysis. Surprisingly, attachments of \(\text{HeT}-A\) occurred significantly less frequently than those of TART elements in spn-\(E\)2 mutants. No \(\text{HeT}-A\) additions were observed in the lines carrying spn-\(E^{\text{Hls3987}}\) and aub\(^{\text{Q42}}\) 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 \(\text{HeT}-A\) 3′ termini [5′-CCAGCAAGTTA-3′] were conserved and terminated by short oligo(A)\(_{14–11}\) 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 \(\text{HeT}-A\) attachments to a broken chromosome end.

Genetic screening of terminal elongation events in the progeny of individual \(y^{\text{TD}}/y\) ac; spn-\(E^{\text{Hls3987}}\)/MKRS and \(y^{\text{TD}}/y\) ac; aub\(^{\text{Q42}}\)/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 \(\text{HeT}-A\)/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 \(\text{HeT}-A\). 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 \(\text{HeT}-A\) are detected by Southern in \(A^{−}\) genomic DNA of the flies in which PCR analysis earlier revealed TART 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 1. Frequencies of terminal attachments in spn-E and aub mutants**

| RNAi mutations    | F1   | F2   | F3   | F4   | F5   | % Q (F3 − F4) |
|-------------------|------|------|------|------|------|--------------|
| spn-\(E^{I}\)/MKRS | 1/569| 3/146| 41/1281| 13/1162| 11/759| 2.1          |
| spn-\(E^{Hls3987}\)/MKRS | 1/556| 36/416| 18/156| 35/247| 37/998| 6.5          |
| aub\(^{Q42}\)/CyO  | 0/651| 3/306| 10/787| 17/2629| —    | 0.8          |
| Controlb           | 0/735| 0/1005| 0/595| 0/744| 0/824| 0.0          |

\(^{a}\)Q, average frequency of visible events signifying a new yellow phenotype(s) versus total number of flies scored (as a percentage).

\(^{b}\)Combined results of fly score obtained for \(y^{\text{TD}}/y\) ac; TM6/MKRS and \(y^{\text{TD}}/y\) ac; CyO/\(\text{if}\) lines.

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

| Attachment type | RNAi mutations |
|-----------------|----------------|
|                 | spn-\(E^{I}\) | spn-\(E^{Hls3987}\) | aub\(^{Q42}\) |
| TART            | 29             | 38             | 16            |
| \(\text{HeT}-A\) | 6              | —              | —             |
| Total\(^{a}\)   | 35             | 38             | 16            |

\(^{a}\)Number 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/+; spn-Ebhs3987/+ and aub^{2Cag2}+ 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 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{77} chromosome. Approximately 50 mature oocytes, which are rarely produced in ovaries of homozygous spn-E mutants, were dissected from 10 spn-E/+; spn-E 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 TT/y ac; spn-E/+/spn-Ebhs3987 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/+ and spn-E/+bhs3987 oocytes results in a high frequency of HeT-A transpositions to the chromosome end, but no TART attachments were found in these cases.

RNAI controls telomere length

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 yTT/y ac; TM6/MKRS, yTT/y ac; spn-E+/MKRS and yTT/y ac; spn-E+/bhs3987 flies. Since the level of antisense TART and HeT-A transcripts detected by 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 flies and, further, an increase of TART expression in spn-E/+; spn-Ebhs3987 ovaries as compared with yTT/y ac; TM6/MKRS ovaries (Fig. 3A). However, while a statistically significant but small dosage effect of spn-E in HeT-A expression was detected in ovaries of spn-E/+ females, a drastic accumulation of HeT-A transcripts was observed in ovaries of spn-E/+/spn-Ebhs3987 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
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-E1/spn-Ehls3987 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-E1/+ ovaries is faint [Fig. 4A]. This observation is consistent with the increased frequency of TART attachments to the broken chromosome ends in spn-E1/+ 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 dsRNAi-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-E1/+ ovaries and are not observed in ovaries of spn-Ehls3987/+ and aubQC42/+ 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.
This observation argues that TART is a primary target of the RNAi machinery in oocytes. 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 oocytes. 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 oocytes 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 spinelle-E (spn-E) mutations were ru1 st1 spin-E1 e1 ca1/TM3, Sb1 e1 [bearing a point mutation in the helicase domain], and ru1 st1 spin-Ehs3087 e1 ca1/TM3, Sb1 e1 (caused by P-element insertion). The abu mutants were abuhoc5/CyO, abuhoc5/CyO, abuhoc5/CyO, abuhoc5/CyO, and abuhoc5/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 yTD. The yTD chromosome is lethal. The homologous y ac chromosome has a deletion of yellow and achaete loci but not of any vital genes. Two lines, yTD4/y ac and yTD5/y ac, carrying deficiencies terminating in the region 1-1.5 kb upstream of the yellow transcription start site, were used. The yTD4/y ac; Tm6/MKRS females were crossed to y ac; spin-E1/MKRS males. In the progeny, yTD4/y ac; spin-E1/MKRS females were crossed to y ac; spin-E1/MKRS males for five successive generations to determine the termination of RNA with black aristae. The same crossing was used to study the spin-Ehs3087 allele. The yTD4/y ac; Tm6/MKRS line was used as a control. yTD4/y ac; CyO/H1 females were crossed to y ac; abuhoc5/CyO males to obtain yTD4/y ac; abuhoc5/CyO females. The yTD4/y ac; CyO/H1 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′-TTCTATCaACAGGCTGTCACAGGTGT-3′ and 5′-CCTTCgtTAGTTTATTGCGT-3′ corresponding to GenBank sequence Dmu0279 was cloned into pBS SK: HeT-A: A PCR-amplified fragment using primers 5′-TCATCGAGCATACTACGCTACGAGTGTT-3′ and 5′-CCTTCgtTACAGGTGTATCCGTTGTA-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 P2 progeny of individual yTD4/y ac; spin-E1/MKRS, yTD4/y ac; spin-Ehs3087/MKRS or yTD4/y ac; abuhoc5/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′-CGAAAACCAACACAAAAATTG-3′; TART2, 5′-AAGGA TGCCGACACTGCTACT-3′; TART3, 5′-TGGACGCGCCAC AACGTGAAACA-3′; HeT-A-specific primers: A2H, 5′-TTCG CAAATGTTAATGCCCCGCTG-3′; M1, 5′-CTGCTCT CGGTACACCACAGGC-3′; M3, 5′-CCTATATATTGCCACA TACTGTAATACAA-3′; yellow-specific primers corresponding to GenBank sequences X06481 and X04427: y13, 5′-AAT CATGCGCTGCTGCAGTGTT-3′; y12, 5′-ATTGATTTG CTGCCATTGGTC-3′; y5, 5′-CAGGAGGCTCTGCTGATA GAATCG-3′; y9, 5′-GGTTCAGTTGTTGGCATACAGG-3′; y17, 5′-AAGACCGGCGTCACACCAGGTATC3′; 7y, 5′-CTTG CGCGCATGTTAGCTATGAC-3′. PCR fragments were sequenced directly or cloned in pTR19R.

To reveal TART/HeT-A attachments in oocytes of sterile spin-E mutants, genomic DNA was isolated from oocytes of yTD4/y ac; spin-E1/spn-E1 and yTD4/y ac; spin-E1/spn-Ehs3087 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 yTD4/y ac; spin-E1/spn-E1 or yTD4/y ac; spin-E1/spn-Ehs3087 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′-TATGACCATCAGCCACGAC-3′ and 5′-CTGCTAGAGCCAGCCTCCAG-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 9y.

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