Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the *D. melanogaster* germline

Alexei A. Aravin*†, Natalia M. Naumova*†, Alexei V. Tulin*‡, Vasilii V. Vagin*†, Yakov M. Rozovsky* and Vladimir A. Gvozdev*†

Background: The injection of double-stranded RNA (dsRNA) has been shown to induce a potent sequence-specific inhibition of gene function in diverse invertebrate and vertebrate species. The homology-dependent posttranscriptional gene silencing (PTGS) caused by the introduction of transgenes in plants may be mediated by dsRNA. The analysis of *Caenorhabditis elegans* mutants impaired with dsRNA-mediated silencing and studies in plants implicate a biological role of dsRNA-mediated silencing as a transposon-repression and antiviral mechanism.

Results: We investigated the silencing of testis-expressed *Stellate* genes by paralogous *Su(Ste)* tandem repeats, which are known to be involved in the maintenance of male fertility in *Drosophila melanogaster*. We found that both strands of repressor *Su(Ste)* repeats are transcribed, producing sense and antisense RNA. The *Stellate* silencing is associated with the presence of short *Su(Ste)* RNAs. Cotransfection experiments revealed that *Su(Ste)* dsRNA can target and eliminate *Stellate* transcripts in *Drosophila* cell culture. The short fragment of *Stellate* gene that is homologous to *Su(Ste)* was shown to be sufficient to confer *Su(Ste)*-dependent silencing of a reporter construct in testes. We demonstrated that *Su(Ste)* dsRNA-mediated silencing affects not only *Stellate* expression but also the level of sense *Su(Ste)* RNA providing a negative autogenous regulation of *Su(Ste)* expression. Mutation in the *spindle-E* gene relieving *Stellate* silencing also leads to a derepression of the other genomic tandem repeats and retrotransposons in the germline.

Conclusions: Homology-dependent gene silencing was shown to be used to inhibit *Stellate* gene expression in the *D. melanogaster* germline, ensuring male fertility. dsRNA-mediated silencing may provide a basis for negative autogenous control of gene expression. The related surveillance system is implicated to control expression of retrotransposons in the germline.

Addresses: *Department of Animal Molecular Genetics, Institute of Molecular Genetics, Moscow 123182, Russia. †Department of Molecular Biology, Moscow State University, Moscow, Russia.
Present address: ‡Department of Embryology, Carnegie Institute, Baltimore, Maryland 21210, USA.

Correspondence: Vladimir Gvozdev
E-mail: gvozdev@img.ras.ru

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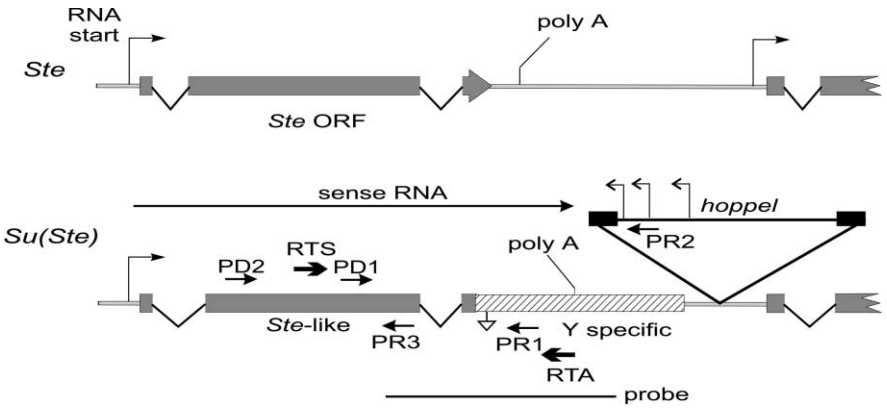
Background

RNA interference (RNAi) refers to sequence-specific inhibition of gene function by its homologous double-stranded RNA (dsRNA). This phenomenon has been observed in a wide range of eukaryotic organisms, including protozoa, *C. elegans*, *D. melanogaster*, and vertebrates [1–4]. The homology-dependent posttranscriptional gene silencing (PTGS) caused by the introduction of transgenes was first observed in plants and later described in fungi (reviewed in [5]). Studies in plants suggest that PTGS may be mediated by dsRNA corresponding to transcribed regions of the gene [6–8]. The species of small 21–25 nt sense and antisense RNA corresponding to silenced genes were discovered and supposed as a component of degradation machinery in PTGS in plants and RNAi in *D. melanogaster* and *C. elegans* [7, 9–11]. In *C. elegans*, the screen of RNAi-deficient mutants was done, and genes required for dsRNA-mediated silencing have been identified [12, 13]. Some of these mutants show activation of different DNA transposons and cause a relief of transgenic repeat cosuppression in the germline [12–14]. The activation of DNA transposon and a retrotransposon was also observed in a *Chlamydomonas* mutant impaired in PTGS [15]. These results and numerous studies in plants strongly implicate the biological role of dsRNA-mediated silencing as a transposon-repression and antiviral mechanism [6, 16].

Here, we report that *Drosophila* tandemly repetitive genes might be naturally regulated by homology-dependent silencing mediated by dsRNA. We studied an interaction of *Stellate* (*Ste*) and *Suppressor of Stellate* (*Su[Ste]*) repeats in the *D. melanogaster* genome. The paralogous testis-expressed *Ste* and *Su(Ste)* tandem repeats are localized in the *D. melanogaster* genome on the X and Y chromosomes,

Figure 1



The structure of *Ste* genes and *Su(Ste)* repeats and the rationale for RT-PCR analysis of *Su(Ste)* RNA. The 1.2 kb *Ste* repeat unit contains an open reading frame with two introns and an intergenic spacer [20]. The closely homologous 2.8 kb *Su(Ste)* unit contains an additional Y-specific sequence (hatched) and the *hoppel* transposon insertion [18]. The *Ste*-like region of *Su(Ste)* repeats includes the promoter region and heavily damaged open reading frame with 90% nucleotide identity with the *Ste* ORF. A part of the sequenced genomic *Su(Ste)* repeats has a 23 bp deletion in the Y-specific region (triangle) [18]. In wild-type males, approximately an equal number of *Su(Ste)* repeats with and without a deletion are presented, while repeats without a deletion mainly remained in the *cry*$^{+}$Y chromosome. The polyadenylated sense *Su(Ste)* transcripts were revealed as a result of the screening of the testes cDNA library [24]. The bar under the diagram indicates a probe used for Northern blot analysis. Reverse transcription using oligo-dT (dT)-, sense (RTA)-, or antisense (RTS)-specific primers was followed by PCR amplification with the *Su(Ste)*-specific primer pair PD1/PR1 flanking intron 2 to distinguish spliced and nonprocessed *Su(Ste)* transcripts. RT-PCR with the primer pair PD1/PR2 was used to test the presence of the *hoppel* sequences in antisense *Su(Ste)* RNA. The PD2/PR3 primer pair was used for the detection of *Su(Ste)* dsRNA in RNaseOne-treated preparation (see Figure 2c). Positions of the start sites of antisense *Su(Ste)* transcripts, according to primer extension analysis and 5′-RACE experiments, are shown by arrows in the *hoppel* body. These start sites are spaced by 173 nt (primer extension mapping) or by 48 and 441 nt (5′-RACE mapping) from the beginning of the left inverted repeat of *hoppel*.

respectively [17–19]. The ORF of the all-sequenced *Stellate* repeats were shown to be maintained by selective pressure [19] and encode protein with a striking homology to the β subunit of protein kinase CK2 [20, 21]. Moreover, in vitro experiments have shown that *Stellate*-encoded protein can interact with the catalytic α subunit of the CK2 enzyme, altering its activity [21]. The hyperexpression of *Stellate* genes is thought to be suppressed by the homologous *Su(Ste)* tandem repeats [18, 20, 22]. Deletion of the bulk of *Su(Ste)* repeats localized in the *crystal* locus of the Y chromosome (*cry*$^{+}$Y chromosome) results in hyperexpression of *Stellate* in testes and causes meiotic abnormalities and accumulation of crystalline aggregates containing *Stellate*-encoded protein in primary spermatocytes [21, 22]. In fly strains containing a high copy number of *Ste* repeats, their hyperexpression, due to the deletion of *Su(Ste)* repeats, caused male sterility [17]. *Su(Ste)* repeats have an *Ste*-like region with about 90% nucleotide identity to the *Stellate* genes in a promoter and coding region with randomly positioned nucleotide substitutions. Each *Su(Ste)* repeat unit also contains a Y-specific region with no sequence similarity to *Stellate* genes and a 1360 (*hoppel*) transposon insertion (Figure 1) [18, 23]. In contrast to *Stellate* genes, all sequenced *Su(Ste)* repeats have damaged open reading frames and are considerably more diverged, suggesting the absence of selective pressure to sustain coding capacity [18, 23]. Sense *Su(Ste)* transcripts with the site of polyadenylation located in a Y-specific region upstream of *hoppel* transposon insertion have been revealed as a result of testes cDNA library screening [24].

Here, we present strong evidence in favor of a homology-dependent RNAi-related mechanism of *Stellate* silencing caused by *Su(Ste)* repeats. We also show that mutation-relieving *Su(Ste)*-dependent silencing of *Stellate* leads to derepression of LTR and non-LTR retrotransposons as well as other genomic tandem repeats in the germline.

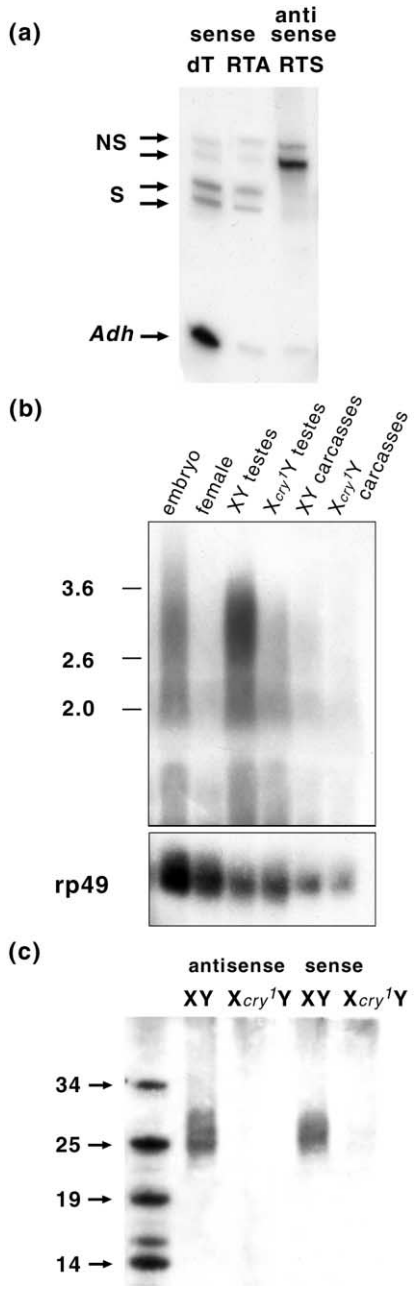
Results

Both strands of *Su(Ste)* repeats are transcribed, producing sense and antisense RNA

To test whether both strands of *Su(Ste)* repeats are transcribed, we used strand-specific RT-PCR and Northern analysis. Figure 1 shows the rationale for RT-PCR analysis. Specific primers were designed to provide strand-specific reverse transcription of the *Su(Ste)* RNA, followed by PCR using primer pairs flanking intron 2 to distinguish the spliced and nonprocessed transcripts. Both unprocessed antisense *Su(Ste)* transcripts as well as spliced, and vestiges of nonspliced, sense *Su(Ste)* transcripts were detected in the testes of males carrying a normal Y chromosome (Figure 2a). Two types of genomic *Su(Ste)* repeats differing by a 23 bp deletion [18] in an amplified region produce antisense as well as sense transcripts. A reverse transcription reaction using oligo-dT primer resulted in the same PCR products, with a great predominance of the spliced form, as was revealed with the sense-specific primer. This result suggests that antisense *Su(Ste)* transcripts failed to enter into an RT reaction with the oligo-dT primer and belong to a nonpolyadenylated RNA fraction.

Northern blot analysis with the *Su(Ste)* strand-specific probe revealed antisense transcripts with an average size

Figure 2



The analysis of *Su(Ste)* transcripts. **(a)** RT-PCR of *Su(Ste)* transcripts. Total testes RNA isolated from wild-type males was used for reverse transcription with oligo-dT (dT)-, sense (RTA)-, or antisense (RTS)-specific primers followed by PCR using the *Su(Ste)*-specific primers PD1 and PR1. Amplification products generated from antisense *Su(Ste)* transcripts as well as spliced (S) and unspliced (NS) sense transcripts are indicated. Pairs of bands correspond to the transcription of genomic *Su(Ste)* repeats differing by a 23 bp deletion (see Figure 1). Both types of genomic repeats produced spliced (RT-PCR products of 349 and 326 nt) and unspliced (RT-PCR products of 408 and 385 nt) sense transcripts as well as unprocessed antisense transcripts. Coamplification with Alcohol dehydrogenase (*Adh*) primers was used to control RNA quantity. The absence of *Adh* amplification in reaction to *Su(Ste)*-specific RT primers confirms the specificity of reverse transcription reactions. No amplification is revealed when reverse transcriptase is omitted (–RT controls, data not shown). **(b)** Northern analysis of antisense *Su(Ste)* transcripts. Total RNA was isolated from embryos, females, testes, and males with excised testes (carcasses). Heterogeneous antisense *Su(Ste)* RNA with an average size of 3 kb is revealed in testes of the wild-type and is practically absent in *cry*^{1}Y males. Hybridization with ribosomal protein 49 (rp49) RNA probe was used as a gel-loading control. **(c)** The detection of short *Su(Ste)* RNAs in testes. Total testes RNA isolated from wild-type (XY) males or males with a deletion of the bulk of *Su(Ste)* repeats (X*cry*^{1}Y) was loaded on a 15% denaturating acrylamide gel, electroblotted to a membrane, and hybridized to partially hydrolized P^{32}-labeled sense or antisense *Su(Ste)* RNA corresponding to the *Ste*-like region. The hybridization signal is observed only in normal XY males. P^{32}-labeled RNA oligonucleotides were used as size markers.

of 3 kb that correspond to about one *Su(Ste)*-repeating unit (Figure 2b). The observed size heterogeneity of antisense *Su(Ste)* transcripts may be due to imprecise starts and/or terminations of transcription. Using a primer corresponding to the *hoppel* transposon sequence, we revealed that, in contrast to sense transcripts, antisense *Su(Ste)* transcripts include *hoppel* transposon sequences (data not shown). The presence of *hoppel* sequences in antisense *Su(Ste)* transcripts indicates that the *hoppel* transposon might be responsible for the initiation of antisense *Su(Ste)* transcription. Mapping of the 5′ end of the antisense *Su(Ste)* transcripts using primer extension and 5′-RACE experiments indicates that antisense transcription starts in different sites of the *hoppel* transposon body (Figure 1).

The RT-PCR, Northern blot analysis, primer extension, and 5′-RACE experiments provide compelling evidence for the existence of antisense *Su(Ste)* transcripts starting in the *hoppel* transposon and extending through the Y-specific sequence into the region with a high sequence identity (90%) with the *Ste* genes.

Stellate silencing associates with the presence of species of small homologous RNAs in testes

We tested the presence of *Su(Ste)* dsRNA in a total testes RNA preparation using treatment by RNase One, which degrades single-stranded RNA, followed by denaturation, reverse transcription using random primers, and PCR amplification. A denaturation-dependent amplification signal in PCR with *Su(Ste)*-specific primers was obtained using RNA isolated from normal males, but it was barely detectable in *cry*^{1}Y males with a deletion of *Su(Ste)* repeats (data not shown). No amplification with *adh*- or *rp49*-specific primers was observed, suggesting that no single-stranded RNA remained after RNase One treatment. This result indicates the presence of *Su(Ste)* dsRNA in the sample. However, dsRNA might be formed as a result of the annealing of sense and antisense strands during the isolation procedure. The presence of endogenous dsRNA in testes suggests that small RNAs that are derived from the dsRNA by endonucleolytic cleavage are being produced [9, 10, 25, 26]. We tested the presence of small

RNA species homologous to *Stellate* and *Su(Ste)* sequences in the total testes RNA. Northern hybridization with sense or antisense *Su(Ste)* RNA probes revealed the presence of heterogeneoous 25–27 nt RNA species in the total RNA isolated from normal males. No small RNAs were detected in *cry*^{1}Y males, suggesting that they are produced from the *Su(Ste)* locus (Figure 2c). Therefore, as in the cases of artificial RNAi [10, 11, 27] and cosuppression in plants [7, 28], *Stellate* silencing is associated with the presence of small homologous RNAs species, presumably produced by *Su(Ste)* dsRNA cleavage.

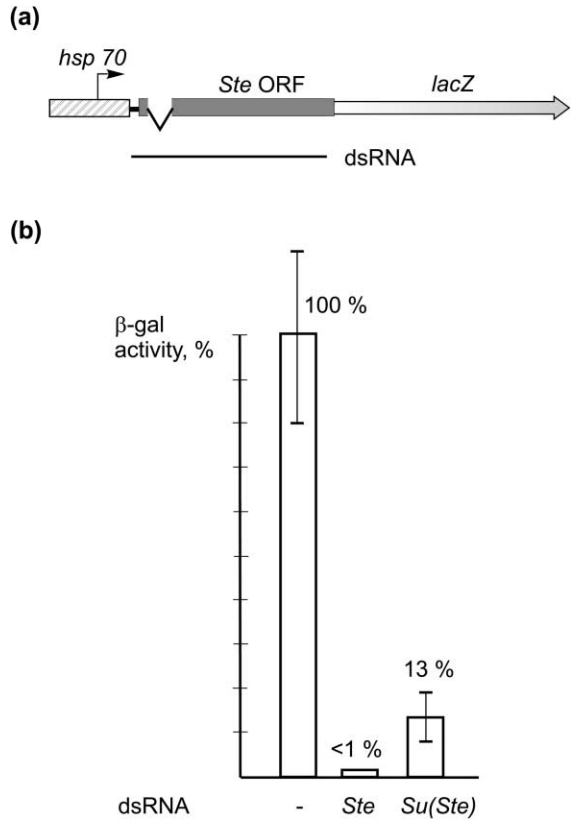
The sequence similarity between *Stellate* and *Su(Ste)* transcripts is sufficient to RNA interference in cell culture

To test the ability of *Su(Ste)* dsRNA to suppress *Stellate* expression, we performed RNAi experiments in *Drosophila* cell culture. Schneider 2 cells were cotransfected with the *hsp70-Ste-lacZ* reporter plasmid and the dsRNA corresponding to *Stellate* or *Su(Ste)* repeats. The reporter construct contains the bulk of *Ste* ORFs fused to the *lacZ* gene (Figure 3a). The expression of the fused *Ste-lacZ* construct was measured by the evaluation of β-galactosidase activity after a heat shock. While *Ste* dsRNA completely abolishes β-galactosidase expression, cotransfection with *Su(Ste)* dsRNA leads to about an 8-fold decrease of expression (Figure 3b). Thus, the extent of sequence identity between *Ste* and *Su(Ste)* repeats is sufficient for strong dsRNA-mediated silencing, although the strength of silencing by *Su(Ste)* dsRNA is significantly lower compared to *Ste* dsRNA. Control experiments show that both *Ste* and *Su(Ste)* dsRNAs do not affect expression of the *hsp70-lacZ* construct carrying no *Ste* sequences.

The short fragment of *Stellate* gene confers *Su(Ste)*-dependent silencing of the *Ste-lacZ* reporter in testes

To reveal the size of a *Stellate* gene fragment that is sufficient to induce *Su(Ste)*-dependent silencing, we used a set of transgene *lacZ* reporters driven by 5′ *Ste* fragments sharing homology to the *Su(Ste)* repeats (Figure 4a). Transgenic flies carrying the *Ste225-lacZ* and *Ste134-lacZ* constructs demonstrate a drastic increase of β-galactosidase expression in the testes of *cry*^{1}Y males as compared to XY males, thus demonstrating a strong response of reporters to the elimination of *Su(Ste)* repeats (Figure 4a,b). The *Su(Ste)*-dependent silencing was observed in all tested transgenic stocks (six and four stocks carrying the *Ste225-lacZ* and *Ste134-lacZ* constructs, respectively), independently of chromosomal localization of insertion. The 134 bp fragment of the *Stellate* gene in the *Ste134-lacZ* construct that is sufficient for establishing the repressed state contains only a 102 bp sequence with 89%–94% nucleotide identity to *Su(Ste)* repeats including 33 bp of 5′-transcribed sequence with *Stellate* ATG start codon fused to the *lacZ* ORF. Thus, a short region of homology to *Su(Ste)* repeats is shown to be sufficient to confer the *Su(Ste)*-dependent silencing. The *Su(Ste)*-dependent silencing of an intron-less construct suggested that the *Ste/Su(Ste)* interaction does not occur on the level of *Ste* transcript splicing, as had been proposed earlier [20].

Figure 3

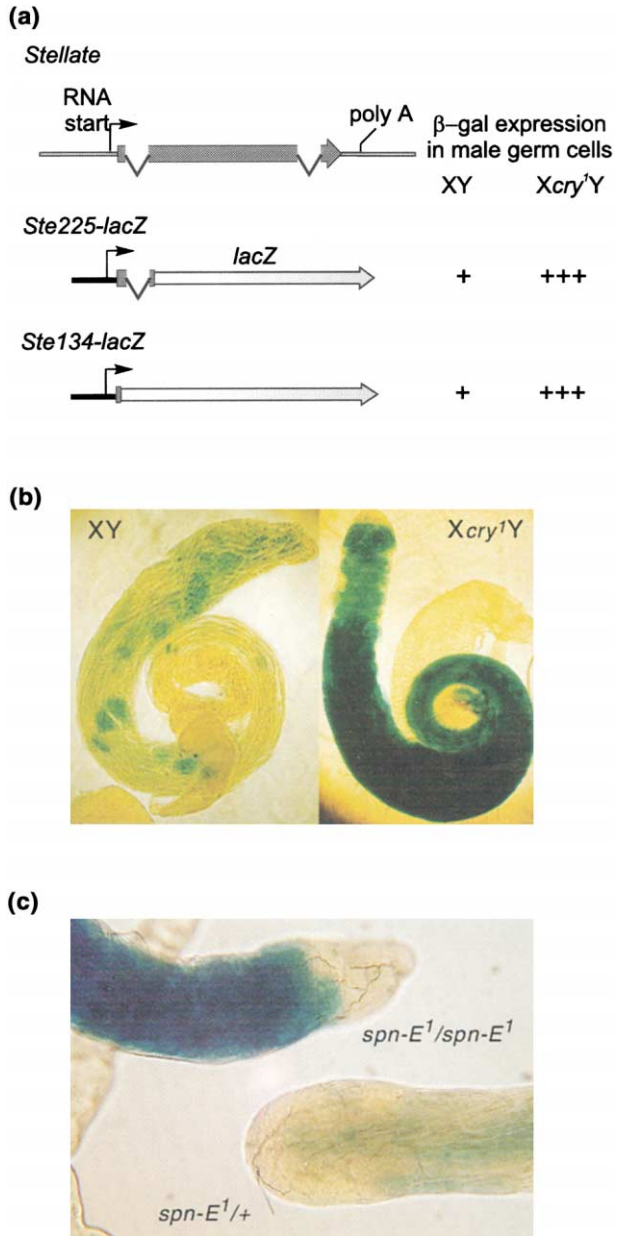


RNAi in *Drosophila* cell culture. **(a)** The reporter *hsp70-Ste-lacZ* construct used in RNAi experiments. The *hsp70* promoter drives expression of the *Ste* ORF fused to *lacZ*. The bar under the diagram indicates a region of dsRNA used in RNAi experiments. **(b)** β-galactosidase expression. Cotransfection with the *hsp70-Ste-lacZ* plasmid and *Ste* dsRNA completely abolishes β-galactosidase expression, while cotransfection with *Su(Ste)* dsRNA leads to about an 8-fold reduction of β-galactosidase activity.

Negative autogenous control of *Su(Ste)* expression

The RT-PCR analysis revealed the presence of *Su(Ste)* antisense transcripts in *cry*^{1}Y males (Figure 5a), encoded by the remnant *Su(Ste)* repeats untouched by the *cry*^{1}Y deletion. Both Northern blot (Figure 2b) and RT-PCR analysis (Figure 5a) show the drastic reduction of antisense *Su(Ste)* RNA in *cry*^{1}Y testes as compared to the wild-type ones. However, in contrast to antisense transcripts, a steady-state level of sense *Su(Ste)* transcripts is significantly increased in *cry*^{1}Y males, despite the deletion of the bulk of *Su(Ste)* repeats (Figure 5b). This observation may be explained by the suggestion that the *Ste/Su(Ste)* interaction is mediated by the *Su(Ste)* dsRNA that targets

Figure 4



The expression of the *Ste-lacZ* reporters in testes of transgenic males. **(a)** The reporter *Ste-lacZ* fusion constructs used for transformation. Different 5′ *Ste* fragments were fused to the *lacZ* ORF. The *Ste225-lacZ* construct comprises 225 bp of *Ste* sequence, including the first intron, but in the *Ste134-lacZ* construct, the *Ste* gene is truncated just downstream of the ATG start codon. Several independent transformant lines carrying insertions of the given construct in different genomic sites were obtained by P-element-mediated transgenesis. **(b)** X-gal staining of testes from transformants, carrying the intronless *Ste134-lacZ* construct in the normal (XY) and X*cry*^{1}Y males. A weak level of β-galactosidase expression, which is greatly increased in testes of *cry*^{1}Y males, is seen in germ cells of XY males. No staining in somatic tissues was observed. **(c)** Expression of the *Ste134-lacZ* construct in *spn-E*^{1} flies. β-galactosidase activity is elevated in the testes of homozygous, as compared to heterozygous, *spn-E*^{1} males.

both *Stellate* and sense *Su(Ste)* transcripts. Therefore, the *cry*^{1}Y deletion, causing a drastic decrease of the *Su(Ste)* dsRNA level (Figure 2c), results in an increase of sense *Su(Ste)* expression. Thus, dsRNA may be considered as a negative autogenous regulator of sense *Su(Ste)* expression. It should be noted that a decrease in the dsRNA level failed to cause an increase of antisense *Su(Ste)* transcripts (see Discussion).

We revealed a weak expression of *Su(Ste)* repeats in heads of adult flies (Figure 5c,d). Both sense and antisense *Su(Ste)* RNA were detected in heads. As in testes, the level of antisense transcripts in heads is drastically decreased in *cry*^{1}Y males (Figure 5c), while the level of sense transcripts is increased (Figure 5d). This observation suggests that the *Ste*/*Su(Ste)*-silencing mechanism also operates in somatic tissues.

aubergine and *spindle-E* mutations cause a relief of *Stellate* and sense *Su(Ste)* silencing

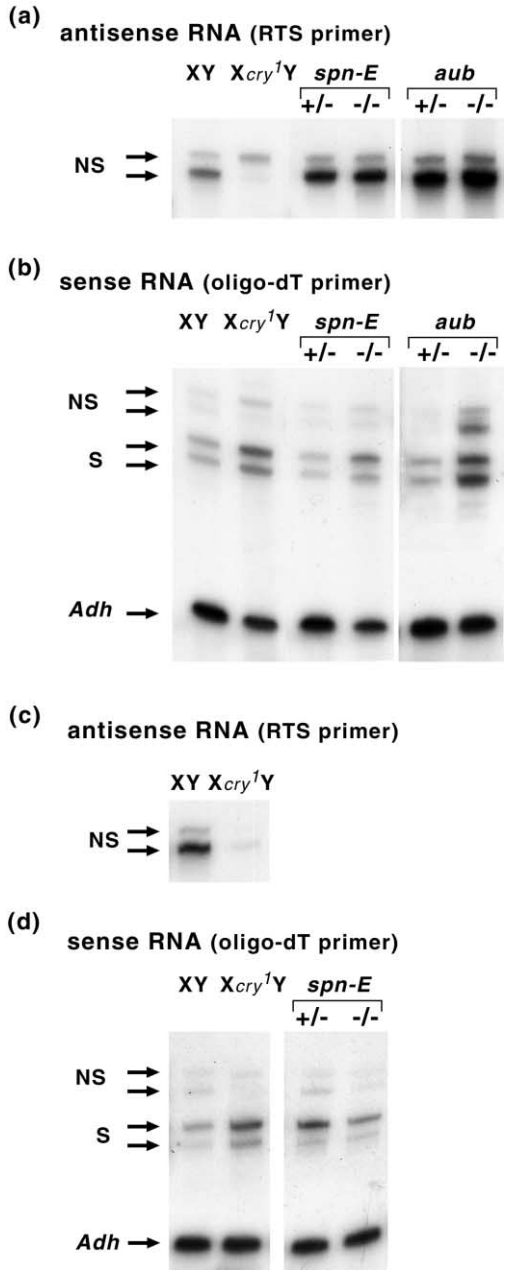
Stellate derepression in the presence of the intact *Su(Ste)* locus has been observed as a result of *aubergine* (*aub*) and *spindle-E* (*spn-E*) mutations, also known as *sting* and *homeless*, respectively [29, 30]. The AUBERGINE protein has homologs involved in PTGS and RNAi in plants, fungi, and animals [12, 31–33]. The *spn-E* gene encodes a putative RNA helicase [34] that is also proposed as a participant in dsRNA-mediated silencing [35].

We confirmed that a relief of *Stellate* silencing occurs as a result of the *spn-E*^{1} mutation by studying the expression of the *Ste-lacZ* reporter construct in the *spn-E*^{1}/+ and *spn-E*^{1}/*spn-E*^{1} males. The expression of β-galactosidase in testes is greatly enhanced in *spn-E*^{1}/*spn-E*^{1} males as compared to the heterozygous ones (Figure 4c). The effects of the *aub*$^{sting-1}$ and *spn-E*^{1} mutations on the level of sense and antisense *Su(Ste)* transcripts were assessed. Both mutations, when homozygous, have no effect on the level of antisense *Su(Ste)* transcripts, but increase the level of sense *Su(Ste)* RNA (Figure 5a,b). Thus, a common mechanism, assisted by the AUBERGINE and SPINDLE-E proteins is operated in *Su(Ste)* dsRNA-mediated silencing of *Stellate* and sense *Su(Ste)* expression. The effect of the *spn-E*^{1} mutation is restricted to the germline, since no increase in the level of sense *Su(Ste)* transcripts in the heads of homozygous flies was observed (Figure 5d).

spn-E mutation leads to derepression of retrotransposons and genomic tandem repeats in the germline

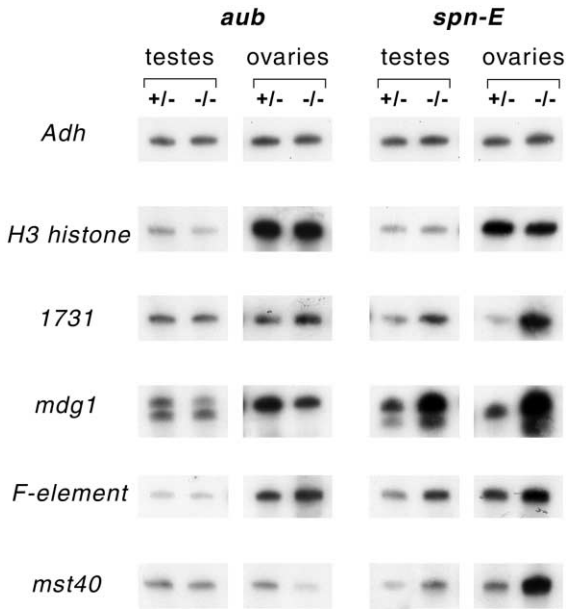
Several, but not all, *C. elegans* mutants that are resistant to dsRNA injection exhibit the mobilization of endogenous DNA transposons and derepression of repetitive transgenes in the germline [12–14]. We compared the steady-state level of polyadenylated transcripts of several transposable elements and nonmobile genomic tandem repeats in hetero- and homozygous flies carrying the *spn-E* and *aub* mutations. The LTR retrotransposons *mdg1* and *1731*

Figure 5



The effect of the *Su(Ste)* deletion and mutations in *aub* and *spn-E* on *Su(Ste)* expression. The RT-PCR procedure was performed as in Figure 2a. The oligo-dT primer and antisense-specific primer (RTS) were used for reverse transcription of sense and antisense *Su(Ste)* transcripts, respectively. **(a)** Antisense *Su(Ste)* expression in testes. Antisense *Su(Ste)* expression is reduced in *cry*^{1}Y males. The genomic *Su(Ste)* repeats without the 23 bp deletion in the amplified region remain predominantly in the *cry*^{1}Y chromosome. The *aub*$^{sting\text{-}1}$ and *spn-E*^{1} mutations have no effect on the level of antisense transcripts. **(b)** Sense *Su(Ste)* expression in testes. The level of sense *Su(Ste)* transcripts is increased in *cry*^{1}Y males, despite the deletion of the bulk of *Su(Ste)* repeats. The *aub*$^{sting\text{-}1}$ and *spn-E*^{1} mutations lead to an increase in the level of sense *Su(Ste)* RNA, when homozygous. **(c)** Antisense *Su(Ste)* expression in heads. **(d)** Sense *Su(Ste)* expression in heads. The level of sense *Su(Ste)* transcripts is increased in the heads of *cry*^{1}Y males. In contrast to testes, the level of sense transcripts is not affected by *aub*$^{sting\text{-}1}$ and *spn-E*^{1} mutations.

Figure 6



The effects of *aub*$^{sting\text{-}1}$ and *spn-E*^{1} mutations on the expression of transposable elements and genomic tandem repeat in the germline. Total RNA from hetero (+/−) and homozygous (−/−) flies was used for reverse transcription with the oligo-dT primer, followed by PCR using primer pairs specific to different repetitive elements. The expression of the LTR retrotransposons (*mdg1*, *1731*), non-LTR retroposon *F-element*, and genomic germline-expressed noncoding tandem repeats *mst40*, as well as the level of histone H3 and Alcohol dehydrogenase (*Adh*) mRNA, is tested. The level of mobile element transcripts and *mst40* tandem repeats was greatly increased in both the male and female germline of homozygous *spn-E*^{1} flies. In the case of *mdg1* transcripts, an additional band is observed in several cases, corresponding to the damaged *mdg1* copy(ies).

as well as the non-LTR *F-element* were chosen for analysis. We also tested the expression of genomic germline-expressed tandem repeats *mst40* [36] and histone H3 transcripts encoded by repeating units [37].

We detected no significant differences in the level of histone H3 expression between the hetero- and homozygous *aub*$^{sting\text{-}1}$ flies in testes and ovaries (Figure 6). The expression of *1731*, *mdg1*, and *F-element* retrotransposons as well as *mst40* tandem repeats was not affected by *aub*$^{sting\text{-}1}$ mutations in testes, but a moderate decrease of *mdg1* and *mst40* expression was detected in ovaries of *aub*$^{sting\text{-}1}$/*aub*$^{sting\text{-}1}$ flies, while the level of *1731* retrotransposon RNA was slightly increased. On the whole, our results suggest that the *aubergine* mutation failed to cause systemic activation

of retrotransposons and genomic tandem repeats in the germline.

In contrast to *aub*$^{sting\text{-}1}$, the levels of transcripts of all tested retrotransposons and *mst40* tandem repeats were significantly increased in both the male and female germline of the *spn-E*^{1} homozygous flies (Figure 6). The similar effect of retrotransposons and *mst40* activation was observed in the male and female germline. The most pronounced effect was detected for the *1731* and *mdg1* expression. Curiously, in ovaries of *spn-E*^{1} homozygous females, the level of *mst40* transcripts was shown to be significantly higher than in testes, although *mst40* transcripts have been originally described as male specific [36]. In contrast to *mst40* and *Ste|Su(Ste)* tandem repeats, the level of histone H3 transcripts was unaffected in both the male and female germline. Thus, SPINDLE-E protein participates in the *Ste|Su(Ste)* interaction as well as in the silencing of different retrotransposons and genomic tandem repeats in the germline.

Discussion

Stellate repeats are silenced by dsRNA generated by the transcription of *Su(Ste)* repeats

The diverged repetitive X-linked *Stellate* and Y-linked *Su(Ste)* gene clusters are involved in balanced interactions sustaining male fertility in *D. melanogaster*. *Stellate* hyperexpression, due to the absence of repressor *Su(Ste)* repeats, is known as a cause of male sterility [17, 20–22]. However, the mechanism of *Su(Ste)*-dependent silencing of *Stellate* genes remains obscure. The presented results allow us to conclude that both sense and antisense transcription of *Su(Ste)* repeats lead to dsRNA formation that is involved in homologous silencing of *Stellate* genes. These results provide the first direct demonstration that *Drosophila* genes might be naturally regulated by homology-dependent silencing mediated by dsRNA.

We detected *Su(Ste)* dsRNA and small 25–27 nt RNA species homologous to *Stellate* and *Su(Ste)* sequences in total RNA preparation isolated from testes of normal, but not *cry*^{1}Y, males carrying a deletion of the bulk of *Su(Ste)* repeats. Thus, the presence of these RNAs is associated with silencing. The 21–25 nt small RNAs are generally assumed to be a hallmark of dsRNA-mediated silencing [9–11, 25–27]. These RNAs have been proposed to guide the endonucleolytic cleavage of a target mRNA bearing a sequence complementary to that of the small RNAs in RNAi in Drosophila [9–11] and *C. elegans* [27]. The small RNA species were also detected in the cases of silencing caused by the introduction of artificial transgenes in plants [7, 28, 38]. It is believed that small RNAs are produced from dsRNA by endonucleolytic cleavage. Recently, DICER, a protein that cleaves the dsRNA to 21–23 nt fragments, was identified in *Drosophila* [26]. The absence of small RNAs in testes of *cry*^{1}Y males carrying a deletion of the bulk of *Su(Ste)* repeats suggests that they are produced by the cleavage of *Su(Ste)* dsRNA. The size of small RNA species in the case of *Stellate* silencing (25–27 nt) is slightly longer then the size of 21–23 nt RNA produced in vitro by dsRNA cleavage in *Drosophila* embryo extracts [9, 25], suggesting that small RNA-producing machinery may differ in some respect. It remains to be elucidated whether DICER or other protein(s) perform *Su(Ste)* dsRNA processing in testes.

We showed that *Su(Ste)* dsRNA produces silencing of the reporter *hsp70-Ste-lacZ* construct in *Drosophila* cell culture, but *Su(Ste)* dsRNA with about 90% nucleotide identity to *Stellate* sequence causes a less profound suppressor effect than the *Ste* dsRNA. This observation is in agreement with the previous studies that demonstrated that the potential of dsRNA to induce silencing drops with a decrease in its sequence identity to a target, with a minimal threshold level of about 85% [11, 27]. Accordingly, the expression of the *Stellate*-related gene β*CK2tes* [39], sharing only 72% sequence identity to *Su(Ste)* repeats, is unaffected by the absence of *Su(Ste)* repeats in XO males [40].

The observation that the silencing of *Stellate* genes is mediated by homologous *Su(Ste)* dsRNA suggests that the same mechanism might be directed to the *Su(Ste)* transcripts. Actually, we revealed that the transcription of *Su(Ste)* repeats leads to a repression of their own sense expression. The level of sense *Su(Ste)* transcripts is increased in spite of the deletion of the bulk of *Su(Ste)* copies, demonstrating a negative mode of autogenous regulation of gene expression. Usually, negative autogenous regulation occurs when the protein gene product regulates transcription of a gene encoding this protein. In our case, negative autogenous regulation is operated by the production of the dsRNA that is supposed to be involved in the elimination of sense transcripts. The loss of a number of *Su(Ste)* copies prevents the accumulation of dsRNA and provides the basis for an increase of sense expression. Possibly, this mechanism of negative autogenous regulation occurs more widely and might operate in the regulation of a unique gene expression.

The *Ste|Su(Ste)* interaction seems to be similar to the cosuppression phenomenon in plants and *C. elegans* in which silencing of both inserted transgenes and homologous endogeneous genes has been observed [14]. Cosuppression of homologous transgenes was also demonstrated in *Drosophila* [41, 42]. However, in all of the described cases, cosuppression has been caused by artificial gene manipulations. In contrast, the *Ste|Su(Ste)* interaction represents the first case of a naturally occurring cosuppression mechanism.

Previous studies suggest that RNAi can target sense as

well as antisense RNA strands for degradation [11]. However, we observed that only sense *Su(Ste)* expression was upregulated in *cry*^{f}Y males. At the same time, no increase of antisense *Su(Ste)* expression was revealed due to *cry*^{f}Y deletion. Possibly, nonpolyadenylated antisense *Su(Ste)* transcripts are not exported from the nucleus, being less accessible for dsRNA-directed degradation machinery.

Using transgenic flies carrying *Ste-lacZ* reporter constructs, we demonstrated that the 134 bp fragment of the *Stellate* gene, encompassing a 102 bp sequence with 89%–94% nucleotide identity to *Su(Ste)* repeats, is sufficient to confer *Su(Ste)*-dependent silencing. However, only 33 bp of this fragment are transcribed and will be a target for degradation if a posttranscriptional mechanism is operated. The recent observation that a dsRNA as short as 26 bp is still capable of inducing RNA interference in *C. elegans* [27] suggests that it is not impossible. However, the presented evidence of the involvement of *Su(Ste)* dsRNA in silencing does not exclude the possibility of transcriptional silencing caused by dsRNA-driven modification of the *Stellate* chromatin structure. Recent study indicates that dsRNA corresponding to promoter sequences may trigger transcriptional gene silencing in plants, accompanied by the appearance of small 21–25 nt RNA [28]. The involvement of the same chromatin-remodeling and methylation proteins in posttranscriptional and transcriptional gene silencing indicates the interconnection of these mechanisms in plants [43]. Components of RNAi machinery were also shown to be involved in the cosuppression caused by repetitive transgene arrays associated with a change in the chromatin structure of the array in the *C. elegans* germline [14]. Further investigation must address the relationship between posttranscriptional and transcriptional mechanisms in *Stellate* silencing.

aubergine and *spindle-E* control expression of repetitive sequences in the germline

The *aubergine* (*sting*) and *spindle-E* (*homeless*) mutants have been shown to upregulate *Stellate* expression [29, 30]. Here, we confirmed the role of *spn-E* in *Stellate* silencing and showed that both mutations lead to an increase in the level of sense *Su(Ste)* transcripts. We also demonstrated that the *spn-E*, but not *aub*, mutation increases the expression of different retrotransposons and genomic tandem repeats.

Both *aub*- and *spn-E*-encoded proteins control translation and localization of specific mRNAs during oogenesis [34, 44]. The AUBERGINE protein is homologous to *C. elegans* RDE-1 [12], *Neurospora crassa* QDE-2 [32], and *Arabidopsis* AGO-1 [31] proteins. All of these proteins have been shown to be involved in RNAi and PTGS phenomena. Recently, the PAZ- and piwi-conserved protein domains that are shared by numerous proteins, including AUBERGINE, RDE-1, QDE-2, and AGO-1, that are implicated in gene silencing and stem-cell maintenance in plants, fungi, and animals were described, but the precise biochemical function of these proteins is unknown [33]. The DICER protein containing the PAZ domain was shown to operate in the cleavage of dsRNA to 21–23 nt fragments in the *Drosophila* cell culture [26]. *Spindle-E* encodes putative RNA helicase with the DExH domain [34]. RNA helicase has been postulated as a component of dsRNA-mediated silencing machinery that functions in dsRNA unwinding to provide sequence-specific target recognition [35]. Recently, the *smg-2* gene encoding the RNA helicase involved in the nonsense-mediated decay pathway has been shown to be required for the persistence of RNA interference in *C. elegans* [45].

Studying the role of *aub* and *spn-E* mutations in the relief of *Stellate* silencing, we observed that these mutations increase the level of sense *Su(Ste)* transcripts, exerting no effect on antisense transcripts. This observation suggests that corresponding proteins are involved in the mechanism of silencing downstream of antisense RNA production. The expression of *spn-E* is restricted to the germline [34]. Accordingly, no influence of *spn-E* mutation on the expression of *Su(Ste)* was detected in heads. However, an increased level of sense *Su(Ste)* transcripts in heads of *cry*^{f}Y males compared to normal XY males indicates that other proteins might participate in *Su(Ste)* dsRNA-mediated repression in somatic tissues. This conclusion is in agreement with recent findings that the RNAi effect in somatic tissues of *D. melanogaster* may be produced by transgene-encoded dsRNA [46].

The expression of the *aubergine-lacZ* reporter construct has been detected only in a tip of testis in which the stem cells are situated [29], but expression of the *Ste-lacZ* reporter is observed in all germ cells of testes, except for a tip (Figure 4b). If both reporter constructs reflect the natural expression patterns of *aub* and *Stellate*, then their expression areas are not spatially overlapping in testes. These results argue that AUBERGINE may participate in the earlier stage of the establishment of silencing and may be dispensable for the later steps, as has been shown for its *C. elegans* homolog, RDE-1, in RNAi [47].

There is ample evidence to implicate the operation of a host surveillance system after mobile elements and viruses [6, 7, 12, 13, 16, 48, 49] as a natural function of dsRNA-mediated silencing. Homologous RNA-mediated silencing of the non-LTR retroposon I-element has been demonstrated in *D. melanogaster*, and an involvement of dsRNA has been proposed [50–53]. We show that the *spn-E*, but not *aubergine*, mutation causes derepression of the *mst40* genomic tandem repeats and a wide spectrum of non-LTR and LTR-containing retrotransposons in the *D. melanogaster* germline. The *spn-E* encoding putative RNA helicase inhibits the expression of repetitive ele-

ments, which may be considered as selfish, but exerts no effect on essential repetitive histone genes. Recently, it was demonstrated that a mutation in the *mut6* gene encoding DEAH RNA helicase impaired PTGS in *Chlamydomonas* and led to an increase in the steady-state level of transposable element transcripts by preventing their degradation [15]. The AUBERGINE protein is involved in dsRNA-mediated *Stellate* repression, but seems to be unrelated to the silencing of retrotransposons and other genomic repeats in the germline. This observation is in accord with a report that the relief of transposon silencing was not observed in mutants of the *rde-1* gene, the *C. elegans* homolog of *aubergine* involved in RNAi [12].

Double-stranded RNA may be considered as a signal for recognition and silencing of repetitive elements in a genome. Applying the technique we used to detect the *Su(Ste)* dsRNA, we obtained preliminary results suggesting that the transcription of several *D. melanogaster* retrotransposons could potentially lead to the formation of dsRNA in the germline. Together with the observation of increasing retrotransposon transcript levels in the *spn-E* homozygous flies, this result suggests that retrotransposon expression in the *Drosophila* germline is controlled by the mechanism that is related, but not identical, to dsRNA-mediated silencing of *Ste/Su(Ste)* repeats. Antisense transcripts of mobile elements may be produced by a read-through mechanism from promoters of adjacent genes or by an internal antisense promoter. Intriguingly, the dsRNA-mediated mechanism of *Stellate* suppression might have evolved as a result of *hoppel* DNA transposon insertion in the genomic *Su(Ste)* repeats, leading to antisense transcription. The transcription of both sense and antisense strands, which provides a potential source of dsRNA, has been shown for numerous *Drosophila* transposable elements, including *mdg1* retrotransposon [54] and the *F-element* [55].

Recent reports of RNAi experiments in mammals [4, 56] suggest the possibility that related silencing mechanisms of repetitive genes might exist in vertebrates. One may speculate that dsRNAs are implicated in the silencing of repetitive genes, since it has been shown that heterogeneous nuclear ribonucleoprotein (hnRNP) particles isolated from mammalian cells contain dsRNA and a significant part of these duplexes is represented by repetitive sequences [57, 58]. The future studies of RNAi-related phenomenon may result in unexpected findings, uncovering the role of dsRNA-mediated silencing in genome surveillance, especially in germ cell development.

Materials and methods

RT-PCR

The amplification conditions were determined in preliminary experiments using plasmid templates. The specificity of the *Su(Ste)* PD1/PR1 primer pair was confirmed by the absence of amplification using female genomic DNA. Total RNA was isolated from 100 hand-dissected testes using Trizol reagent (Gibco BRL), followed by two sequential precipitations with LiCl at a final concentration of 3 M, quantified by absorbancy at 260 nm, and treated with Amplification Grade DNaseI (Gibco BRL) according to manufacturer's instructions. Samples were divided for +RT and –RT reactions. Oligo-dT primer (0.5 μg) or 2 pmol of *Su(Ste)* strand-specific primers RTS (5′-TGCACCGAAARTATATG-3′) or RTA (5′-AAT GAGTGCTTGATATGA-3′) were added to total RNA (1 μg) and heated to 70°C for 10 min. First strand cDNA was synthesized with SuperScript II reverse transcriptase (200 U; Gibco BRL) for 1 hr at 43°C. Controls without RT were processed in parallel. The enzyme was heat inactivated at 70°C for 15 min. Reverse transcription of several independently isolated RNA samples was performed. Samples (2 μl) from the reverse transcription reaction were amplified using Taq polymerase in the presence of 0.027 pM/μl 4 μCi/pM αP^{32} dATP. To detect *Su(Ste)* transcripts, the following primers were used: PD1 5′-CCCAGCTKYCCGGA CATCTTCTT-3′, PD2 5′-GGCATGATTCACGCCCGATACAT-3′, PR1 5′-CTTGGACCGAACACTTTGAACCAAGTATT-3′, PR2 5′-AGTGGC TGCATAGTGCCAAACCAAT-3′, and PR3 5′-AGGGGCGATCTCA AGTTCG-3′. Primers used in the RT-PCR of other transcripts correspond to the following sequences: *mdg1*, 875–900 and 1026–1051 nt in GenBank sequence S68526; *F-element*, 1321–1347 and 1532–1558 nt in sequence M17214; *1731*, 1138–1153 and 1257–1271 nt in sequence X07656; *mst40*, 194–218 and 416–441 nt in sequence Z22588; histone H3, 3522–3547 and 3731–3753 nt in sequence X14215; and *Adh*, 6183–6202 and 6380–6399 nt in sequence Z00030. The linear range of amplification was determined in preliminary experiments, and 22–26 cycles were performed, depending of transcript abundance. Amplification was performed at annealing temperature of 60°C. Coamplification with Alcohol dehydrogenase (*Adh*) primers was used to control RNA quantity. Samples (10 μl) of the PCR reaction were loaded on a 5% denaturating acrylamide gel, and products were visualized by autoradiography after 2 hr–overnight exposure to X-ray film.

Detection of Su(Ste) *dsRNA*

Total RNA was isolated from 100 hand-dissected testes using Trizol reagent (Gibco BRL). Total RNA (10 μg) was digested with 1.5 U of RNaseOne (Promega) in a total volume of 50 μl for 10 min at 37°C. The reaction was quenched by the addition of 1.5 μl 10% SDS, followed by ethanol precipitation in the presence of yeast tRNA. After resuspension in 50 μl of 1× DNaseI buffer, the samples were treated with 5 U DNaseI (Ambion) and 2 U RNaseH (Amersham) for 1 hr at 37°C. Reactions were quenched by the addition of 10 μl DNase inactivation reagent (Ambion) according to manufacturer's instructions. For denaturation, samples were incubated for 10 min at 97°C. Aliquots without denaturation were processed in parallel. Samples (10 μl) were used in 20 μl reverse transcription reaction with 250 μg/ml of random primers (Promega) and 200 U of SuperScript II reverse transcriptase (Gibco BRL). Reactions were incubated for 10 min at 25°C and then 1 hr at 43°C. RT reactions (2 μl) was used in PCR with the PD2/PR3 primer pair. At the annealing temperature of 60°C, 25 cycles were performed in the presence of 0.027 pM/μl 4 μCi/pM αP^{32} dATP. Samples of the PCR reactions were loaded on a 5% denaturating acrylamide gel, and products were visualized by autoradiography during overnight exposure to X-ray film.

Detection of small RNA species

The detection of small RNA was essentially performed by following the protocol of Hutvagner et al. [38]. Briefly, 50 μg total testes RNA isolated using Trizol reagent was loaded in each lane of a 15% denaturating acrylamide gel, electrophoresed, and electroblotted to Hybond N+ membrane (Amersham) using 0.5× TBE buffer. ^{32}P-labeled riboprobe was transcribed by T7 RNA polymerase (Boehringer) using the *Su(Ste)* fragment as a template, producing a sense or antisense RNA probe for hybridization. After synthesis, the labeled RNA was partially hydrolyzed during 1 hr incubation at 60°C in the presence of 80 mM $NaHCO_3$, 160 mM Na_2CO_3. Hybridization was performed in 25% formamide, 0.5 M NaCl, 0.1 M Na_2HPO_4, 25 mM EDTA, 1× Denhardt solution, and 150 μg/ml denaturated DNA at 50°C. After hybridization, a membrane was washed twice in 2× SSC, 0.5% SDS at 50°C for 30 min and once in 0.5× SSC, 0.5% SDS at 50°C for 15 min. Overnight exposure to X-ray film was sufficient to detect a signal from small RNA.

Primer extension and 5′-RACE

Primer extension with Ext primer (5′-AACTGGCAGTGCATTT-3′) was performed on 20 μg total testes RNA, essentially following the protocol of Livak [20]. 5′-RACE experiments using RTS primer for reverse transcription and PD1 primer for amplification were performed using the SMART RACE cDNA amplification kit (Clontech) according to the protocol of Matz et al. [59]. PCR products were cloned and sequenced.

Northern hybridization

Total RNA (20 μg) was loaded on 1% agarose 3-[N-morpholino]propanesulfonic acid (MOPS) formaldehyde gel, electrophoresed, and transferred to Hybond N membranes (Amersham). A ^{32}P-labeled riboprobe was transcribed by T3 RNA polymerase (Boehringer) using the *Su(Ste)* fragment as a template (shown in Figure 1) inserted into pBlueScript SK, thus producing a sense RNA probe for hybridization. Hybridization and washing were done according to standard procedures. As a control, hybridization with a rp49 probe was used.

Reporter construction design

For PCR amplification, a plasmid template containing six full-length *Ste* genes was used. The 134 bp *Ste* fragment for the *Ste134-lacZ* construct was amplified using the 5′-GAGTCTAGAGTTCCCATCTGGAAGGG CAT-3′ and 5′-TAGGATCCATGTTGCCAGTTCACTTGTTCACAGAAA TATG-3′ primer pair, digested with XbaI and BamHI, and ligated into the CaSpeR-β-gal vector [60] opened with XbaI and BamHI. For the *hsp70-Ste-lacZ* construct, the 592 bp *Ste* fragment was amplified using the 5′-GAGAATTCATATTTCTGTGAACAAGTGAACTGGCA-3′ and 5′-ACGGATCCAGGGGCGATCTCAAGTTCG-3′ primer pair, digested with EcoRI and BamHI, and ligated into the CaSpeR-β-gal vector downstream of the previously cloned *hsp70* promoter. The *Ste225-lacZ* construct comprises *Ste* sequences from 298 to 523 bp, comprising GenBank sequence X97141, fused to the β-galactosidase ORF in the CaSpeR-β-gal vector. The selected clones were sequenced to confirm correct fusion.

Drosophila *strains, transformation, and genetic crosses*

P-element-mediated germline transformation of *Df(1)w$^{67c23(2)}$y* embryos was performed according to the standard protocol [61]. The strain with a deletion of the bulk of *Su(Ste)* repeats on the Y chromosome was *cry^{1}*Y, described in Palumbo et al. [22] To produce males carrying the *cry^{1}*Y chromosome, *Df(1)w$^{67c23(2)}$y* females were crossed to X/*cry^{1}B^{s}Yy^{+}* males. Strains carrying *aubergine* and *spindle-E* mutations were *y^{1} ac^{1} sc^{1} w^{1} Ste^{+}*, P{*lacW*}*aub$^{sting-1}$*/*Cy* and *Ste^{+}*, and *ru^{1} st^{1} spn-E^{1} e^{1} ca^{1}*/TM3, *Sb^{1} e^{s}*, respectively.

RNAi experiments in Drosophila *cell culture*

RNAi experiments essentially followed the protocol of Hammond [10]. The Schneider 2 cells were cultured at 25°C in culture media containing 10% heat-inactivated fetal bovine serum (FBS). The cells were cotransfected at densities of 2×10^6 cells per ml with dsRNA (1.2 μg per ml of cell culture) and *hsp70-Ste-lacZ* plasmid (6 μg per ml of cell culture) by calcium phosphate coprecipitation [62]. The expression of a reporter construct was induced 48 hr after transfection by 20 min of 37°C heat shock. After 2 hr of relaxation at 25°C, cells were lysed in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1mM $MgSO_4$, 50 mM β-mercaptoethanol, and 0.5% NP40), and the expression of β-galactosidase was assessed by ONPG (O-Nitrophenyl-β-D-galactopyranoside, Sigma) staining [63]. For dsRNA production, transcription templates were generated by polymerase chain reaction on *Stellate* or *Su(Ste)* plasmid templates such that they contained T7 promoter sequences on each end. The dsRNA was prepared using T7 RNA polymerase (Boehringer) according to manufacturer's instruction. After transcription, the RNA was heated to 65°C for 10 min, then cooled to room temperature for 1 hr. To monitor annealing, RNAs were electrophoresed in a 2% agarose gel in TBE buffer and stained with ethidium bromide.

X-gal staining of testes

Hand-dissected testes were fixed in 2% glutaraldehyde in PBS for 30 min, washed two times in PBS, and stained with 0.25% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) at 37°C for 1.5 hr in buffer containing 0.15 M NaCl, 10 mM NaH_2PO_4 (pH 7.5), 1 mM $MgCl_2$, 3.1 mM $K_3[Fe_{II}(CN)_6]$, and 3.1 mM $K_4[Fe_{III}(CN)_6]$.

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