MOLECULAR AND CELLULAR BIOLOGY, Aug. 2004, p. 6742–6750 Vol. 24, No. 15
0270-7306/04/$08.00+0 DOI: 10.1128/MCB.24.15.6742–6750.2004

Dissection of a Natural RNA Silencing Process in the *Drosophila melanogaster* Germ Line

Alexei A. Aravin,[1]† Mikhail S. Klenov,[1] Vasilii V. Vagin,[1] Frédéric Bantignies,[2] Giacomo Cavalli,[2] and Vladimir A. Gvozdev[1]*

Department of Animal Molecular Genetics, Institute of Molecular Genetics, Moscow 123182, Russia,[1] and Chromatin and Cell Biology Laboratory, Institute of Human Genetics, Centre National de la Recherche Scientifique, 34396 Montpellier, France[2]

Received 12 November 2003/Returned for modification 31 December 2003/Accepted 6 April 2004

To date, few natural cases of RNA-silencing-mediated regulation have been described. Here, we analyzed repression of testis-expressed *Stellate* genes by the homologous *Suppressors of Stellate* [*Su*(*Ste*)] repeats that produce sense and antisense short RNAs. The *Stellate* promoter is dispensable for suppression, but local disturbance of complementarity between the *Stellate* transcript and the *Su*(*Ste*) repeats impairs silencing. Using in situ RNA hybridization, we found temporal control of the expression and spatial distribution of sense and antisense *Stellate* and *Su*(*Ste*) transcripts in germinal cells. Antisense *Su*(*Ste*) transcripts accumulate in the nuclei of early spermatocytes before the appearance of sense transcripts. The sense and antisense transcripts are colocalized in the nuclei of mature spermatocytes, placing the initial step of silencing in the nucleus and suggesting formation of double-stranded RNA. Mutations in the *aubergine* and *spindle-E* genes, members of the Argonaute and RNA helicase gene families, respectively, impair silencing by eliminating the short *Su*(*Ste*) RNA, but have no effect on microRNA production. Thus, different small RNA-containing complexes operate in the male germ line.

The silencing of genes by homologous double-stranded (ds) RNA (RNA interference [RNAi]) was discovered in artificial systems where dsRNA is introduced by injection or by expression of transgenic constructs (11, 25, 43). RNAi is widely used as a powerful technique for switching off specific genes and as a tool of whole-genome screening (12, 22). The first case of natural dsRNA-mediated silencing was found in *Drosophila melanogaster*, where suppression of a euchromatic locus by homologous testis-expressed heterochromatic repeats has been shown to be necessary for male fertility (2, 26, 27). In testes of wild-type males, hyperexpression of the X-linked *Stellate* genes is prevented by the closely homologous, bidirectionally transcribed, Y-linked *Supressor of Stellate* [*Su*(*Ste*)] repeats (2, 26), and deletion of *Su*(*Ste*) leads to abnormalities of spermatogenesis (7, 35).

Twenty-one- to 23-nucleotide (nt) short interfering (si) RNAs formed by processing of long dsRNA molecules by the RNase Dicer play a central role in dsRNA-mediated silencing (4, 46, 55). siRNA is assumed to act as a guide for the RNase complex (RISC) that degrades homologous mRNAs (9, 17, 33). microRNAs are involved in the control of expression of cellular genes (14, 19, 20, 29). microRNAs are transcribed as precursors with a stem-loop structure that are processed by Dicer into single-stranded RNAs of a size similar to that of siRNA. Recent experiments suggest that siRNAs and microRNAs can be loaded into a common RISC (20, 28, 29, 32). The Argonaute family of proteins represents the conserved core component of siRNA- and microRNA-containing complexes isolated from both *D. melanogaster* and mammals (18, 21, 29, 32).

The repression of *Stellate* in *D. melanogaster* by the closely homologous *Su*(*Ste*) repeats shares a number of traits with artificial RNAi. Bidirectional transcription of *Su*(*Ste*) repeats leads to formation of short sense and antisense RNAs (2). Derepression of *Stellate* occurs in *aubergine* (*aub*) and *spindle-E* (*spn-E*) mutants, genes that encode an Argonaute-family protein and a DExH RNA helicase, respectively (2, 40, 44). Recently, a requirement for the Aub and Spn-E proteins for dsRNA-injection-provoked RNAi was shown in *D. melanogaster* embryos (23). In the fission yeast, *Schizosaccharomyces pombe*, components of the RNAi machinery are involved in natural repression of centromeric heterochromatin, which contains remnants of transposable elements that are transcribed bidirectionally (51). Short RNAs complementary to centromeric repeats have been found (38), and mutations in genes encoding the Argonaute protein and the dsRNA-processing enzyme Dicer lead to derepression of centromeric repeats (15, 51). Antisense transcription of *Su*(*Ste*) repeats in *D. melanogaster* is thought to be caused by a *hoppel* transposon insertion (2). Thus, two systems of natural silencing, one in *D. melanogaster* and one in *S. pombe*, evolved as a result of heterochromatic genome rearrangements and use of the RNAi machinery.

Here, we extended the study of natural dsRNA-mediated silencing in *D. melanogaster*. Using in situ hybridization, we examined the order of steps that lead to *Su*(*Ste*) dsRNA formation. We found that (i) accumulation of antisense *Su*(*Ste*) RNA is followed by formation of dsRNA in the nuclei of spermatocytes at successive stages of spermatogenesis, (ii) mutations in the *aub* and *spn-E* genes lead to disappearance of short *Su*(*Ste*) RNA, but (iii) these mutations have no effect on

* Corresponding author. Mailing address: Institute of Molecular Genetics of RAS, Kurchatov sq., 2, Moscow 123182, Russia. Phone: (7-095)196-0012. Fax: (7-095)196-0221. E-mail: gvozdev@img.ras.ru.
† Present address: Laboratory for RNA Molecular Biology, The Rockefeller University, New York, NY 10021.

microRNA formation, indicating that different protein complexes are involved in the formation of microRNA and *Su*(*Ste*) short RNA in testes. We also show that the *Stellate* promoter is dispensable for silencing and propose a posttranscriptional mechanism for *Stellate* repression.

MATERIALS AND METHODS

Reporter construct design. A plasmid template containing six full-length *Ste* genes was used for PCR amplification. For the *Ste134mut-lacZ* construct, the 134-bp *Ste* fragment was amplified by using a 5′-GAGTCTAGAGTTCCCATC TGGAAGGGCAT-3′ and 5′-TAGGATCCATGTTGCCAGTTCTGATGTTC ACAGAAATATG-3′ primer pair, digested with XbaI and BamHI, and ligated into the CaSpeR-β-galactosidase (β-Gal) vector (47) opened with XbaI and BamHI. For the β2*tub-Ste-lacZ* construct, the 592-bp *Ste* fragment was amplified by using a 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 β2*tub* promoter. The selected clones were sequenced to confirm correct fusion.

***Drosophila* strains, transformation, and genetic crosses.** P-element-mediated germ line transformation of *Df (1)w*$^{67c23(2)}$, *y* embryos was performed according to standard protocol (39). For remobilization of the *P*{*Ste134mut-lacZ*} element, the *yw; Sb[2-3]/TM6B* stock was used. The number of insertions was determined by Southern blot analysis. The *cry*^{1}*Y* strain, with a deletion of most *Su*(*Ste*) repeats, has been described (35). To produce males carrying the *cry*^{1}*Y* chromosome, *Df (1)w*$^{67c23(2)}$, *y* females were crossed to *X/B*^{s} *cry*^{1}*Y*y^{+} males. The strains carrying the *aub* and *spn-E* mutations were *y*^{1} *ac*^{1} *sc*^{1} *w*^{1} *Ste*$^{+}$; *P*{*lacW*}*aub*$^{sting-1}$/*Cy* and *Ste*$^{+}$; *ru*^{1} *st*^{1} *spn-E*^{1} *e*^{1} *ca*^{1}/TM3, *Sb*^{1} *e*^{s}.

Detection of transcripts by in situ hybridization. DNA fluorescent in situ hybridization (FISH) experiments on whole-mount embryos was performed as described previously (3). A plasmid template containing a full-length *Stellate* gene (1,150 bp) was used for PCR amplification to produce 631-bp PCR products containing a *Stellate* open reading frame and either T3 or T7 RNA polymerase promoter sequences. PCR products were cleaned before transcription, using a PCR purification kit (QIAGEN), and probes were transcribed by T7 or T3 RNA polymerase. About 1 μg of template was used in a 10- to 20-μl transcription reaction mixture with a nucleotide mix containing either digoxigenin (DIG)-UTP or biotin-UTP. The labeled RNA was partially hydrolyzed by incubation at 60°C in a 40 mM $NaHCO_3$–60 mM Na_2CO_3 solution. After neutralization and ethanol precipitation, RNA was dissolved in 20 μl of water and 80 μl of hybridization solution HS (50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus0.015 M sodium citrate], 0.1% Tween 20, 5 mg of torula RNA/ml, 50 μg of heparin/ml) was added.

Testes were dissected in 1× phosphate-buffered saline (PBS), fixed for 20 min in 4% paraformaldehyde in 1× PBS, washed three times for 5 min in PBT (1× PBS, 0.1% Tween 20), treated with a solution of 50 μg of proteinase K/ml in 1× PBS for 8 min, washed with a solution of 2 mg of glycine/ml in PBT for 2 min and two times for 5 min in PBT, refixed for 20 min in 4% paraformaldehyde in 1× PBS, and washed two times for 5 min in PBT and finally with 50% HS in PBT. After prehybridization in HS at 60°C for 1 to 3 h, samples were hybridized overnight at 60°C in 150 to 200 μl of HS containing 2.5 to 5 ng of riboprobe/μl. After hybridization, samples were washed three times for 30 min in HS at 60°C, 15 min in 50% HS in PBT at 60°C, two times for 15 min in 2× SSC–0.1% Tween 20 at 60°C, two times for 15 min in 0.2× SSC–0.1% Tween 20 at 60°C, and two times for 15 min in PBT at room temperature. Samples were incubated for 1 to 2 h in 1× PBS–0.3% Triton X-100–1% bovine serum albumin–10% goat serum for blocking and in the same solution with antibodies for 1 h. The following antibody concentrations were used: 1:2,000 for anti-DIG-alkaline phosphatase (AP) (Roche), 1:50 for anti-rhodamine (Roche), and 1:500 for anti-biotin-fluorescein isothiocyanate (Vector). Samples were washed two times for 15 min in 1× PBS–0.3% Triton X-100, once for 15 min in PBS–250 mM NaCl–0.2% NP-40–0.2% Tween 20, and two times for 15 min in 1× PBS–0.3% Triton X-100.

For fluorescence detection, DNA was counterstained with 0.2 μg of 4′,6′-diamidino-2-phenylindole (DAPI)/ml in PBT for 10 min, washed in PBT, washed in 1× PBS, and mounted in 30 to 40 μl of ProLong antifade (Molecular Probes). For AP reactions, samples were washed for 10 min in AP buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris [pH 9.5], 0.1% Tween 20) and incubated with 1 ml of AP buffer with 20 μl of nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) stock solution (Roche) added. Development of the reaction was observed visually, and the reaction was stopped after 0.5 to 1 h. Samples were washed two times for 3 min with PBT and mounted in 70% glycerol in 1× PBS.

For DNA and RNA FISH experiments, images were acquired with a cooled charge-coupled device camera (Micromax YHS 1300; Roper Scientific) mounted on a DMRXA Leica microscope with a 100× Plan/Apo objective (numerical aperture, 1.4) mounted on a Roper Scientific piezo electric *z*-axis actuator. For Fig. 2, single slices from *z* stacks were deconvolved by a Huygens MLE single-TIF procedure (Scientific Volume Imaging).

dsRNA processing in vitro. PCR products of the *lacZ* or *Su*(*Ste*) gene carrying T7 promoters on both ends were used as transcription templates. RNA was transcribed by using T7 RNA polymerase (Boehringer) according to the manufacturer's instructions. RNA was heated to 65°C for 10 min and then slowly cooled to room temperature for 1 h. Cell lysates of Schneider 2 cells were prepared as described previously (4). To obtain testis extract, 50 testis pairs were dissected on ice in PBS solution and centrifuged at 6,000 × *g*, PBS was removed, and an equal volume (25 μl) of buffer was added (25 mM HEPES [pH 7.4], 100 mM CH_3COOK, 2 mM $MgCl_2$, 6 mM β-mercaptoethanol, 1 mg of AEBSF protease inhibitor and Complete protease inhibitor [Roche]/ml). Tissue grinder homogenization was followed by centrifugation at 20,000 × *g* for 20 min. Internally [α-^{32}P]UTP-labeled dsRNA (50 nM) was incubated at 25°C for 1 h in a 50-μl reaction volume containing 25 μl of lysate, 1 mM ATP, 10 mM creatine phosphate, and 30 μg of creatine phosphokinase/ml. Reactions were stopped by the addition of Trizol reagent (GIBCO BRL). RNA was isolated by using the standard Trizol protocol and was analyzed by electrophoresis in a 15% polyacrylamide gel. [γ-^{32}P]ATP-labeled RNA size markers were prepared by using T4 polynucleotide kinase (New England Biolabs).

β-Gal activity assay. Eight pairs of hand-dissected testes were added to 200 μl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.35% β-mercaptoethanol). Testes were homogenized with a tissue grinder, and 100 μl of 0.4% ONPG (*o*-nitrophenyl-β-D-galactopyranoside) (Sigma) in Z buffer was added. Samples were incubated at 37°C for 3 h, and the reaction was stopped by adding 1 ml of 0.52 M Na_2CO_3. The extracts were centrifuged at 20,000 × *g* for 1 min. β-Gal activity was calculated from absorbance measured at 420 and 550 nm.

Detection of *Su*(*Ste*) small RNA and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining of testes were performed as described previously (2). The detection of miR-304 and miR-12 was carried out by hybridization (30) with the oligonucleotides CTCACATTTACAAATTGAGATTA and ACCAGTACC TGATGTAATACTCA, respectively, labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase (New England Biolabs).

RESULTS

Nuclear localization of *Stellate* and *Su*(*Ste*) transcripts in germ cells. To determine the subcellular localization of *Stellate* and *Su*(*Ste*) transcripts, we used in situ hybridization with sense and antisense *Stellate* single-stranded RNA probes. These probes hybridize to both *Stellate* and *Su*(*Ste*) RNAs due to a high level of sequence identity between them. The specificity of hybridization was first tested by DNA FISH to salivary gland polytene chromosomes and to embryonic interphase nuclei (data not shown). The probe produces a single signal on polytene chromosomes at 12D on the X chromosome, where the euchromatic *Stellate* locus is located. Because heterochromatin is underrepresented in polytene chromosomes, the Y-linked *Su*(*Ste*) locus and the second X-heterochromatin *Stellate* locus are not detected. Hybridization to embryonic interphase nuclei, in contrast, yields two signals in nuclei of female embryos and three signals in male nuclei. These results are consistent with hybridization with the two X-linked *Stellate* loci (in the euchromatin at 12D and in the heterochromatin at h26) and the Y-linked *Su*(*Ste*) locus. Since females carry two X chromosomes that are paired in most somatic nuclei in embryos at this developmental stage, two signals per nucleus are expected, while in males a third signal corresponds to the *Su*(*Ste*) locus located on the Y chromosome.

RNA in situ hybridization with single-stranded probes was

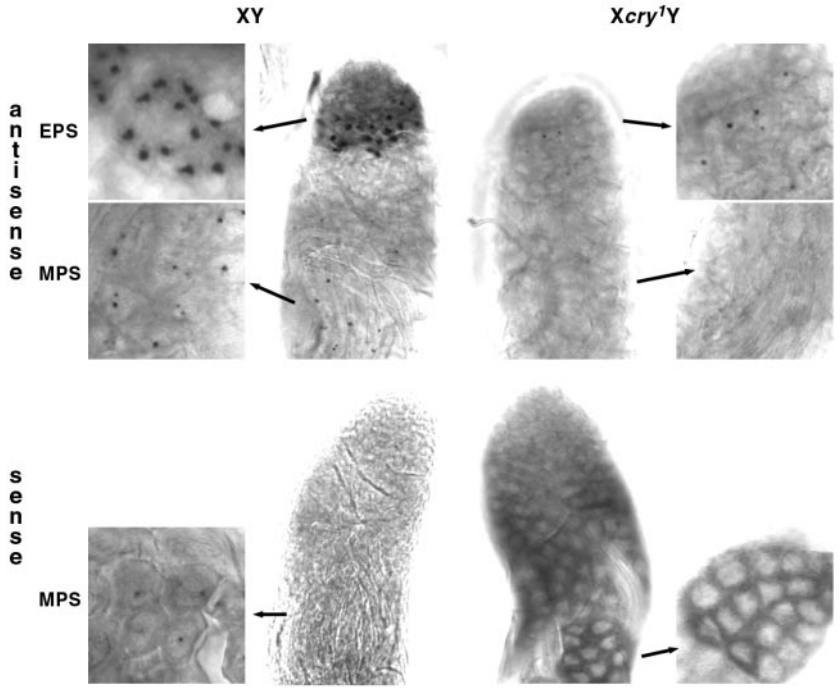

FIG. 1. Localization of sense and antisense *Stellate* and *Su*(*Ste*) transcripts in testes. Sense and antisense RNA were detected by using single-stranded probes in whole testes of wild-type males (XY) and of males with a deletion of the bulk of *Su*(*Ste*) repeats (*Xcry*^{1}*Y*). DIG-labeled probes were visualized by using AP-coupled antibodies following color reaction with NBT/BCIP substrate. The segments shown in enlarged panels correspond to early (EPS) and mature (MPS) primary spermatocyte stages. In wild-type males, antisense transcripts are abundant in the nuclei of early primary spermatocytes, while in *cry*^{1}*Y* males the signal area is greatly decreased. In mature primary spermatocytes, antisense transcripts are detected as one or two sharp dots per nucleus. In *cry*^{1}*Y* males the signals are not visible in all nuclei. Sense RNA is detected only at the mature primary spermatocyte stage, as faint dots in nuclei of the wild-type males or as a strong diffuse signal in the cytoplasm of *cry*^{1}*Y* males.

done on dissected testes, using the same sequences as for the DNA probe described above. *D. melanogaster* testes are composed of male germ cells in successive stages of spermatogenesis and several types of somatic cells. We detected abundant antisense transcripts in early and mature primary spermatocytes. In early primary spermatocytes of wild-type males, the antisense RNA had a diffuse nuclear localization (Fig. 1 and 2a). DNA counterstaining by DAPI showed that the antisense signal corresponds to the non-DAPI-stained nuclear region (Fig. 2a). In the subsequent mature primary spermatocyte developmental stage, strong nuclear antisense signals are seen as distinct dots (one, two, or occasionally more) in each nucleus. No antisense transcripts are detected in the cytoplasm of spermatocytes at any stage. As expected for *cry*^{1}*Y* males, which have a partial deletion of *Su*(*Ste*) repeats, probing for antisense RNA produces a dramatically weaker signal than in wild-type males. Thus, the abundant antisense transcripts of wild-type males have a nuclear localization and their expression is significantly decreased in *cry*^{1}*Y* males.

The probe for *Stellate* and *Su*(*Ste*) sense RNAs does not detect any signal in early primary spermatocytes, which represent the developmental stage where antisense transcripts are first detected. In the subsequent mature primary spermatocyte stage, we detected sense RNA signals in nuclei as distinct faint dots that are qualitatively similar to the signals of antisense transcripts at the same stage (Fig. 1 and 2a). No signal above background was seen in the cytoplasm of wild-type males. In contrast, when silencing was relieved in *cry*^{1}*Y* males, the sense RNA was detected as a strong and dispersed signal in the cytoplasm of mature primary spermatocytes. No sense RNA was detected in the nuclei of *cry*^{1}*Y* males (Fig. 1). Thus, sense transcripts appear after antisense RNAs and are localized as compact dots in nuclei of mature primary spermatocytes of wild-type males but are found in the cytoplasm when silencing is relieved.

We have ruled out the possibility that the sense and antisense RNA signals in nuclei of mature primary spermatocytes arise from DNA hybridization: no signals are detected in somatic cells, and RNase treatment of fixed testes before hybridization eliminates the signals. Simultaneous detection of sense and antisense RNAs demonstrates that the sense and antisense transcripts are colocalized in nuclei of wild-type mature primary spermatocytes (Fig. 2b). This suggests that the sites where sense and antisense transcripts colocalize might represent nuclear sites of dsRNA formation.

The short *Su*(*Ste*) RNA detected in vivo is longer than siRNAs. *Stellate* silencing has been shown to be associated with the presence in testes of a homologous short 25- to 27-nt RNA that is likely processed from *Su*(*Ste*) dsRNA. The short *Su*(*Ste*) RNA is longer than canonical siRNA (21 to 23 nt) and endogenous microRNAs (21 to 24 nt) processed from hairpin precursors in *D. melanogaster* (1). This apparent size difference is not a technical artifact caused by decreased RNA mobility because of a high concentration of total RNA in the samples (data not shown), nor is the length of the short RNA related to some peculiarity of the *Su*(*Ste*) RNA sequence. In vitro-syn-

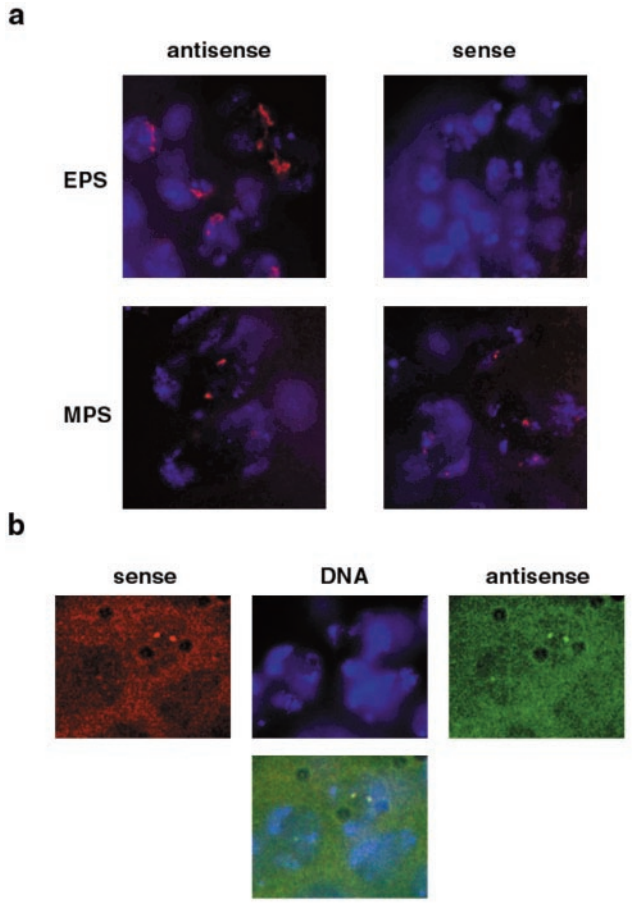

FIG. 2. FISH detection of sense and antisense transcripts in wild-type spermatocytes. (a) Sense and antisense RNAs in the nuclei of early (EPS) and mature (MPS) primary spermatocytes of wild-type males. DIG-labeled probes were visualized by using rhodamine-coupled antibodies (red); DNA was stained with DAPI (blue). Staining in the cytoplasm is due to tissue autofluorescence. (b) Simultaneous detection of sense and antisense transcripts in mature primary spermatocyte nuclei. The DIG-labeled probe for sense RNA was visualized with rhodamine-coupled antibodies (red), and the biotin-labeled probe for antisense RNA was visualized with fluorescein isothiocyanate-coupled antibodies (green). DNA was stained with DAPI (blue). Images obtained from separate channels (upper line) and the composite image (bottom) are shown.

thesized *Su*(*Ste*) dsRNAs incubated with a cell culture extract are processed into fragments of the same size (21 to 23 nt) as *lacZ* dsRNA (Fig. 3a) rather than the 25 to 27 nt of endogenous *Su*(*Ste*) short RNA (Fig. 3b). Thus, both *Stellate* and *Su*(*Ste*) dsRNAs are processed in vitro into fragments of canonic siRNA sizes, while the in vivo product is longer. The same result was obtained using testis extract (Fig. 3a). It cannot be excluded that specific processing occurs only in germ cells, whereas siRNAs of standard size are produced in the somatic cells. However, the complete absence of longer products in testis extract-treated RNA is in conflict with this explanation.

The Aub and Spn-E proteins are required for the presence of *Su*(*Ste*) short RNA in vivo but are dispensable for dsRNA processing in vitro. *Stellate* derepression in the presence of an intact *Su*(*Ste*) locus has been observed as a result of mutations in the *aub* and *spn-E* genes, which encode an Argonaute family protein and a DExH RNA helicase, respectively (2, 40, 44). The *Stellate* derepression caused by mutation was not a result of lack of *Su*(*Ste*) antisense transcription (2). Both *aub* and *spn-E* are required for artificial RNAi in oocytes and embryos (23). *spn-E* mutation also leads to an increased steady-state level of several retrotransposon transcripts in the germ line (2, 44). We tested for the presence of *Su*(*Ste*) short RNA in testes of *aub* and *spn-E* mutant males. *Su*(*Ste*) short RNAs of both polarity are absent in total testis RNA isolated from homozygous *aub* and from homozygous *spn-E* males (Fig. 4a). This observation supports the correlation between *Stellate* silencing and the presence of short RNAs and implicates the Aub and Spn-E proteins in the formation and/or stabilization of the short RNAs. We have also investigated the effect of the *aub* and *spn-E* mutations on the expression of microRNA in testes. The expression of two microRNAs (miR-304 and miR-12) is

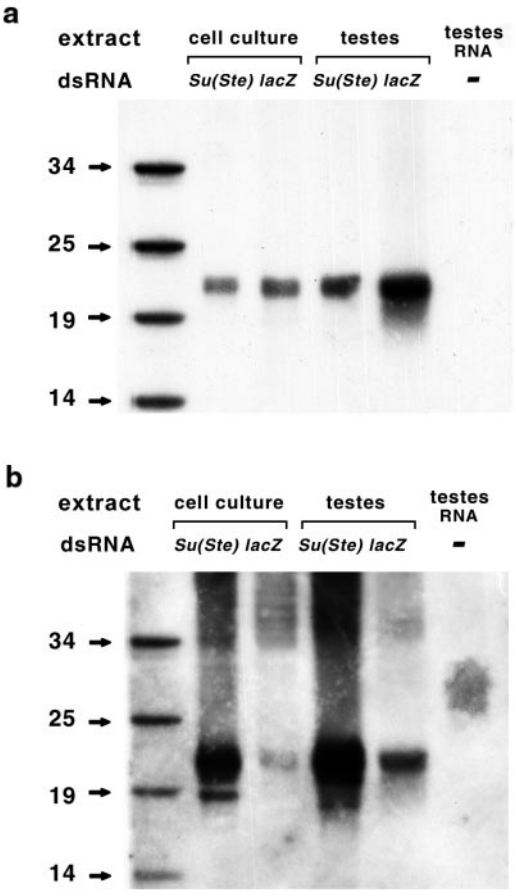

FIG. 3. Sizes of short RNAs produced by dsRNA processing in vitro and detected in vivo. (a) Twenty-one- to 23-nt RNA fragments of *lacZ* and *Su*(*Ste*) are produced by dsRNA processing in cell culture and testis extracts. In vitro-synthesized, uniformly labeled *lacZ* or *Su*(*Ste*) dsRNAs were incubated with cell culture or testis extracts, and RNAs were isolated and separated on a 15% denaturing acrylamide gel. Total testis RNA from wild-type males was separated in parallel. ^{32}P-labeled RNA oligonucleotides were used as size markers. (b) The same gel was electroblotted to a membrane and hybridized with a *Su*(*Ste*) probe to detect the 25- to 27-nt *Su*(*Ste*) RNA in the total testis RNA preparation. Hybridization also increased the strength of signals from the in vitro-synthesized *Su*(*Ste*) dsRNA.

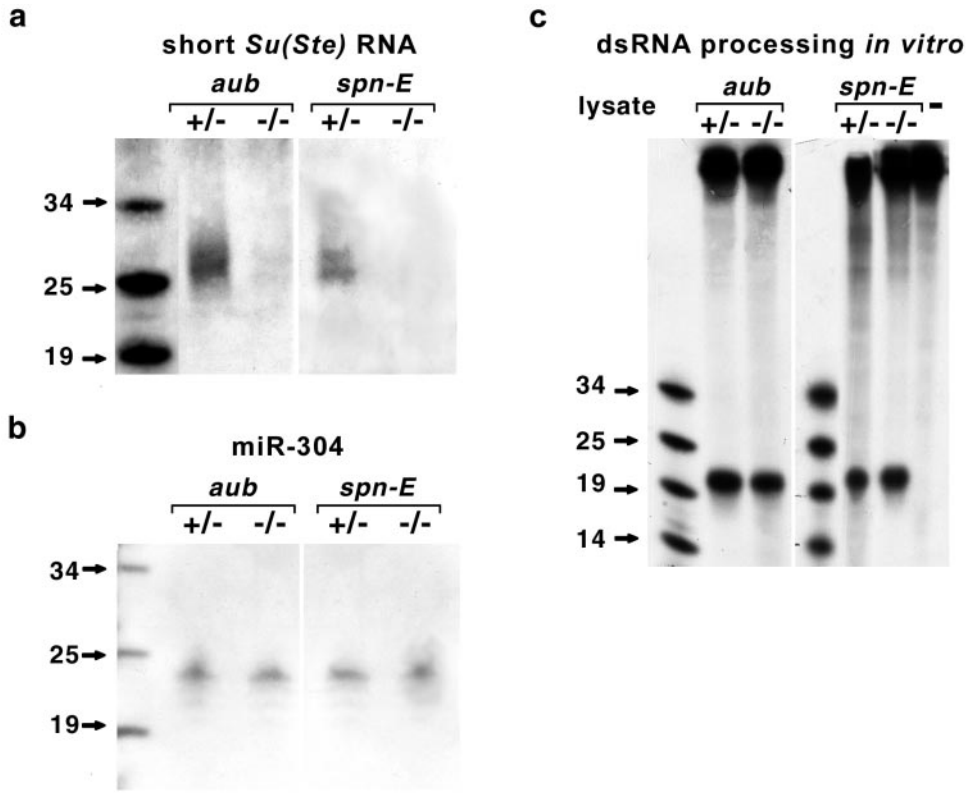

FIG. 4. Effects of *aub* and *spn-E* mutations on the presence of the short *Su*(*Ste*) RNA and of microRNA in vivo and on processing of dsRNA in vitro. (a) Short *Su*(*Ste*) RNAs are absent in homozygous *aub* and *spn-E* mutants. Equal quantities of total RNA isolated from testes of heterozygous (+/−) and homozygous (−/−) males were separated on a gel and hybridized with a *Su*(*Ste*) probe. (b) There is no effect of either mutation on the amount of miR-304. (c) *aub* and *spn-E* mutations do not affect in vitro processing of dsRNA to 21- to 23-nt fragments. *lacZ* dsRNA was incubated with extracts prepared from testes of heterozygous (+/−) or homozygous (−/−) males. No degradation of dsRNA was detected in the absence of testis extract (lysate−).

increased in testes as compared to somatic tissues (data not shown). However, the amounts of both microRNAs were not affected by the *aub* and *spn-E* mutations (Fig. 4b; data not shown). Thus, different sets of proteins are involved in the expression of *Su*(*Ste*) short RNA and microRNAs in testes.

To analyze the role of Aub and Spn-E in dsRNA processing in vitro, ^{32}P-labeled *Su*(*Ste*) dsRNA was incubated with mutant testis extracts. Normal processing of dsRNA into 21- to 23-nt siRNA was observed in testis extracts obtained from both *aub* and *spn-E* males (Fig. 4c), suggesting that the proteins are not directly involved in dsRNA cleavage even though they are required for in vivo production of the 25- to 27-nt *Su*(*Ste*) RNA.

Disruption of homology to *Su*(*Ste*) repeats in the transcribed region of the *Stellate* gene relieves silencing of reporter constructs. *Stellate* silencing may be investigated by using reporter constructs that include *Stellate* sequences fused to *lacZ* (2). The *Ste134-lacZ* construct contains 134 bp of the *Stellate* gene, including 104 bp of nontranscribed sequence followed by 30 bp from the 5′-UTR of the first *Stellate* exon (Fig. 5a). This fragment drives *Su*(*Ste*)-dependent *lacZ* expression in testes (2). Assuming a posttranscriptional mechanism of silencing, only 30 bp of this sequence represents a target for homologous recognition and degradation. We substituted three sequential nucleotides located in the middle of the 30-bp sequence (+16 to +18 with respect to the transcription start) to produce the *Ste134mut-lacZ* construct (Fig. 5a). This mismatch may prevent the complementary interaction between *Stellate* and *Su*(*Ste*) short RNAs. Measurement of β-Gal activity in testes of wild-type males carrying this mutated construct showed a considerable (two- to fivefold for different stocks) increase of *lacZ* expression compared to that in males with the ancestral *Ste134-lacZ* construct (Fig. 5b). In contrast, the expression level was roughly the same for both constructs in *cry^{1}Y* males, suggesting that mutation does not lead to *Su*(*Ste*)-independent promoter activation. Thus, local perturbation of complementarity in the transcribed region between the *Stellate* transcripts and the small *Su*(*Ste*) RNA results in a relief of silencing similar to that produced by *cry^{1}Y* deletion.

To test whether the *Stellate* promoter is necessary for *Su*(*Ste*)-dependent repression, we used a β2*tub-Ste-lacZ* construct. This transgene contains an almost complete *Stellate* open reading frame fused to the *lacZ* gene under control of the heterologous β2-tubulin promoter (Fig. 5a), which provides strong constitutive expression in male germ cells. Weak *lacZ* expression was observed in testes of wild-type males. In *cry^{1}Y* males, however, strong staining was detected in all but the tip of the testes, as expected for β2-tubulin-driven expression (Fig. 5c). In other words, a construct carrying a heterologous promoter that contains only *Stellate* coding sequence is still repressed by *Su*(*Ste*). Hence, the nontranscribed promoter region of *Stellate*

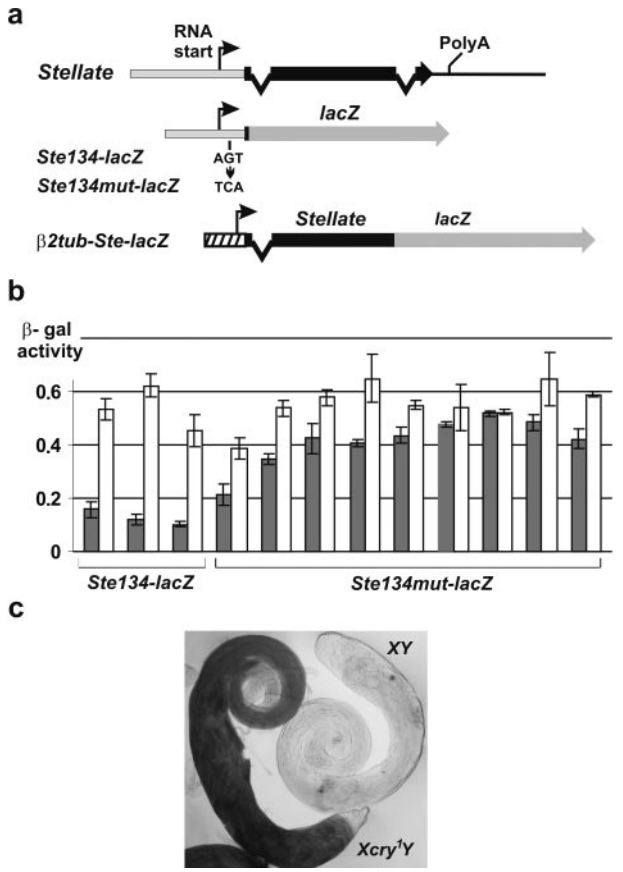

FIG. 5. Structures and expression of reporter constructs containing *Stellate* sequences. (a) The structures of *Stellate* genes and reporter *Ste-lacZ* fusion constructs used for *Drosophila* transformation. The *Ste134-lacZ* construct contains a 134-bp fragment of the *Stellate* gene, including 104 bp of nontranscribed sequence followed by 30 bp of the transcribed noncoding region from the first *Stellate* exon. An arrow indicates the site of substitution of three adjacent nucleotides in the *Stellate* 5′-UTR sequence in the *Ste134mut-lacZ* construct. In the β2*tub-Ste-lacZ* construct, the β2*tub* promoter drives expression of the *Ste* open reading frame, detached from intron 2, fused to *lacZ*. (b) Nucleotide substitutions in the *Stellate* transcribed region result in relief of *Su*(*Ste*)-dependent silencing. β-Gal activity was measured in testis extracts from wild-type (filled bars) and *cry*^{1}*Y* (open bars) males for three stocks carrying independent insertions of *Stel34-lacZ* and eight stocks with independent insertions of *Ste134mut-lacZ*. There is less difference in *lacZ* expression between wild-type and *cry*^{1}*Y* males that carry the *Ste134mut-lacZ* construct than between those that carry the *Stel34-lacZ* construct. Southern analysis confirmed that all of the stocks carry a single transgene insertion. (c) X-Gal staining of testes from transgenic flies carrying the β2*tub-Ste-lacZ* construct. Weak expression was observed in the germ cells of wild-type males, while in *cry*^{1}*Y* males strong staining was detected throughout the testes except in the very tip. *cry*^{1}*Y* males have a five- to sevenfold higher level of β-Gal activity than wild-type (XY) males.

appears to be dispensable for repression, supporting a post-transcriptional mechanism of *Stellate* silencing.

DISCUSSION

Here we present studies of natural RNA silencing of the X-linked *Stellate* repeats. Silencing of *Stellate* repeats in the *D. melanogaster* germ line is required for male fertility and is mediated by an interaction with the homologous heterochromatic Y-linked *Su*(*Ste*) repeats. Natural RNA silencing associated with the presence of short RNA may be a common factor in the control of heterochromatin functions. In *S. pombe* long dsRNA produced from centromeric repeats is processed into short RNAs that guide the initiation of heterochromatin formation (38, 50, 51). Various types of transposable elements that make up a considerable part of the heterochromatin in higher eukaryotes have been shown to be repressed by an RNA-silencing mechanism (1, 8, 24, 45, 48, 53, 56). As a first step in dissecting the *Stellate* silencing mechanism, we have examined the processing and distribution of *Stellate* and *Su*(*Ste*) RNAs.

Nuclear step of dsRNA maturation. Our group has previously shown that *Stellate* gene transcription yields only sense transcripts, while *Su*(*Ste*) repeats yield both sense and antisense transcripts. We have also observed that expression of *Stellate* and sense *Su*(*Ste*) transcripts is repressed in wild-type males but that antisense *Su*(*Ste*) transcripts escape silencing despite their complementarity to short RNAs (2). Here, we showed that antisense RNAs accumulate in the nucleoplasm and are not transported into the cytoplasm. This result supports our earlier proposal that nonpolyadenylated antisense RNAs escape the cytoplasmic degradation machinery because they are sequestered in the nucleus (2).

Sense transcripts are localized in nuclei of mature wild-type primary spermatocytes. In *cry*^{1}*Y* males, in which the *Stellate* genes are derepressed, these transcripts are found only in the cytoplasm. These results correspond to the accumulation of the *Stellate*-coded protein as crystalline aggregates in the cytoplasm of mature primary spermatocytes of *cry*^{1}*Y* males (7). The total amount of *Stellate* and *Su*(*Ste*) sense transcripts is greatly increased in *cry*^{1}*Y* males (2, 7, 35). The absence of a nuclear signal in *cry*^{1}*Y* males, contrasted with the presence of sense transcripts in wild-type nuclei, therefore suggests that these transcripts are never released from the wild-type nucleus. Nuclear retention of sense transcripts in the wild type might be explained by the interaction between sense and antisense transcripts. Nuclear localization of sense and antisense transcripts has also been observed for bidirectionally transcribed *white* transgenes, which induce RNAi of the endogenous *white* gene (13).

The distinct sharp dots observed in the nuclei for both sense and antisense RNAs in mature primary spermatocytes may correspond to the accumulation of the native transcript at the sites of transcription. The signals are often located at the border between the chromatin (DAPI stained) and the nucleoplasmic areas of the nucleus, where actively transcribed loci are thought to be located. Restricted nuclear signals corresponding to the sites of transcription have been observed for a number of genes (52), whereas transcripts in the process of export from the nucleus are usually below the detection sensitivity of the standard in situ hybridization technique. The colocalization of the sense and antisense transcripts suggests the formation of dsRNA in the nucleus, thus placing the initiation of *Stellate* silencing in the nucleus. We propose that these nuclear dsRNA species may involve hybrids between sense and antisense *Su*(*Ste*) transcripts, as well as between sense *Stellate* and antisense *Su*(*Ste*) transcripts, and that these hybrids are essential for *Stellate* silencing by *Su*(*Ste*).

Different sizes of the short *Su*(*Ste*) RNAs and typical siRNAs. We observed a strong correlation between *Stellate* silencing and the presence in testes of sense and antisense 25- to 27-nt RNAs homologous to *Stellate* and *Su*(*Ste*) sequences. The short RNAs are absent when *Stellate* genes are derepressed as a consequence of either a *Su*(*Ste*) locus deletion or mutations in the *aub* and *spn-E* genes. The cloning of short RNA from *D. melanogaster* testes also demonstrates the presence of short RNAs that are derived from *Su*(*Ste*) and are highly homologous to *Stellate* (1). A rigid size restriction of 21 to 23 nt has, however, been observed for siRNA in various in vitro studies of *D. melanogaster* RNAi. Examination of Dicer activity with different dsRNAs suggests a strong specificity of processing to 21- to 23-nt fragments in both *Drosophila* embryo extracts and cell culture (4, 9). Furthermore, investigation of the functional anatomy of chemically synthesized siRNAs in embryo extracts defined the optimal length of siRNAs as 21 to 23 nt, while RNAs longer than 24 nt have practically no cognate-mRNA cleavage activity (10). It has been proposed that only RNAs that meet this size requirement can be loaded into the RISC. However, examples of the existence of two size classes of short RNAs (21 or 22 nt and 24 to 26 nt) involved in silencing have also been reported. Two different size variants of short RNAs were observed during artificial silencing in plants, with the short variant responsible for posttranscriptional gene silencing and the long one most likely participating in DNA methylation and spreading of the silencing signal (16). Furthermore, only RNAs from the long class have been detected that correspond to endogenous plant transposable elements. Two size classes of short RNAs are produced from dsRNA in plant extracts, and the activity of different Dicer proteins was shown to be responsible for producing each class (46). Cloning of endogenous short RNAs from *D. melanogaster* has also identified two size classes of short RNAs, with the short class (21 to 23 nt) including microRNAs and the long class (24 to 26 nt) comprising sequences derived from transcripts of transposable elements and other repetitive heterochromatic sequences (1).

The larger size of the short *Su*(*Ste*) RNA may be explained by specific sequences affecting dsRNA processing by Dicer or by the presence in testes of specific factors that change the cleavage interval of dsRNA. However, we find that exogenous *Su*(*Ste*) dsRNA is cleaved into 21- to 23-nt siRNA in testis extracts, most likely reflecting the activity of the same Dicer protein that acts in somatic tissues. We favor the hypothesis that the 25- to 27-nt *Su*(*Ste*) RNAs detected in vivo are produced by a mechanism at least partially different from conventional siRNA production. A clue to the origin of the short *Su*(*Ste*) RNAs comes from the finding that *Su*(*Ste*) dsRNA formation occurs in the nucleus, unlike that of artificial RNAi, in which dsRNA is believed to be processed in the cytoplasm (5). Both conventional-size siRNA and a longer short RNA have been observed during viroid replication in the plant nucleus (36). Two size classes of short RNAs may be produced in *D. melanogaster* by different Dicer proteins, as has been demonstrated in plants (46). Alternatively, specific nuclear factors may affect how a single Dicer protein processes dsRNA in the nucleus.

Role of Aub and Spn-E in formation of short RNAs. We observed that mutations in the *aub* and *spn-E* genes lead to elimination of short *Su*(*Ste*) RNA in testes. However, neither mutation affects processing of exogenously provided dsRNA to 21- to 23-nt siRNA in testis extracts. It has been observed that both *aub* and *spn-E* mutations block RNAi in oocytes produced by injected dsRNA (23). The authors proposed that both proteins affect RNAi because of their involvement in translational control, but our results suggest that Aub and Spn-E may be involved in the production and/or stabilization of siRNA. Similarly, the *rde-1* and *mut-7* genes of *Caenorhabditis elegans* are required for the production of siRNA in vivo but are dispensable for dsRNA processing in vitro (37, 48). The authors showed that the corresponding proteins are required for long-term stabilization of siRNA rather than for dsRNA processing.

The *aub* and *spn-E* mutations eliminate the short *Su*(*Ste*) RNA without affecting the abundance of two different microRNAs in testes. We propose that distinct protein complexes mediate production and/or stabilization of short *Su*(*Ste*) RNA and microRNAs in testes. Similarly, different members of the Argonaute family participate in artificial RNAi and in microRNA processing in *C. elegans* and plants (5, 16), despite the central role of Dicer in both processes (6, 14).

Mechanism of *Stellate* repression. Homologous silencing mediated by short RNA may occur by posttranscriptional degradation of mRNA (49, 54) and by DNA and chromatin modification leading to transcriptional repression (16, 41, 56). The nuclear antisense RNA accumulation and dsRNA formation that we have found raises the question of whether posttranscriptional or transcriptional mechanisms of silencing operate in *Stellate* repression. For animals, it is generally believed that artificial RNAi caused by dsRNA leads to posttranscriptional degradation of mRNA. However, it has been shown that dsRNA or short RNA can affect transcription and chromatin structure of homologous sequences in plants and *Saccharomyces cerevisiae* (30, 51, 56). In plants, for example, transcriptional silencing of reporter constructs can be caused if the dsRNA produced by hairpin constructs or virus infection is homologous to the untranscribed promoter region of the target gene, while posttranscriptional degradation of the corresponding mRNA occurs if there is homology between the dsRNA and the transcribed sequence (30, 31).

We have observed that constructs containing the *Stellate* coding sequence driven by a heterologous promoter are regulated by *Su*(*Ste*) repeats in the same manner as native *Stellate* genes or reporter constructs with *Stellate* sequence fused to *lacZ*. In contrast, expression of the endogenous β*Nac*-like genes, having a putative promoter region with high levels of sequence similarity (95%) to *Stellate* but an unrelated transcribed sequence, shows no response to the deletion of *Su*(*Ste*) (L. Usakin and G. L. Kogan, unpublished results). In the present study, we also found that nucleotide substitutions in the transcribed region of a *Stellate* fragment homologous to the *Su*(*Ste*) sequence lead to a release of silencing. Thus, homology to *Su*(*Ste*) in the untranscribed region is dispensable for repression, while local disturbance of complementarity in the transcribed sequence impairs silencing. We cannot rule out the possibility that regulatory sequences important for transcriptional silencing may be present in the transcribed region, but our results are more simply explained by a posttranscriptional *Stellate* silencing mechanism.

The two blocks of tandemly repeated *Stellate* genes are located in intercalary and constitutive heterochromatin of the X chromosome (27, 42), and *Su*(*Ste*) repeats are located in the heterochromatic Y chromosome. siRNA-mediated transcriptional repression of centromeric heterochromatin repeats has been recently demonstrated in *S. cerevisiae* (51). Our results do not exclude participation of transcriptional repression of genomic *Stellate* repeats acting in concert with a posttranscriptional mechanism. Similarly, both transcriptional and posttranscriptional mechanisms have been shown to operate in the repression of multicopy transgenes associated with the presence of homologous short RNA in *D. melanogaster* (34). Thus, both transcriptional and posttranscriptional mechanisms might act in *Stellate* silencing, and further studies will be directed to understanding the contribution of each of them.

ACKNOWLEDGMENTS

We thank Leonard Robbins, Yair Dorsett, and Phillip Zamore for text improvements and critical comments.

This work was supported by grants from the Russian Foundation for Basic Researches (N 02-04-48498), INTAS (N 01-0279), the Russian Foundation for Science School Support (N 2074, 2003.4), the Physics-Chemical Biology Program of RAS, and INTAS young scientist fellowship YSF 00-243 to A.A.A. The work of F.B. and G.C. was supported by grants from the CNRS (ATIPE), the Association pour la Recherche sur le Cancer, and the Human Frontier Science Program Organization.

REFERENCES

1. **Aravin, A. A., M. Lagos-Quintana, A. Yalcin, M. Zavolan, D. Marks, B. Snyder, T. Gaasterland, J. Meyer, and T. Tuschl.** 2003. The Small RNA profile during Drosophila melanogaster development. Dev. Cell **5:**337–350.
2. **Aravin, A. A., N. M. Naumova, A. V. Tulin, V. V. Vagin, Y. M. Rozovsky, and V. A. Gvozdev.** 2001. Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the D. melanogaster germline. Curr. Biol. **11:**1017–1027.
3. **Bantignies, F., C. Grimaud, S. Lavrov, M. Gabut, and G. Cavalli.** 2003. Inheritance of Polycomb-dependent chromosomal interactions in Drosophila. Genes Dev. **17:**2406–2420.
4. **Bernstein, E., A. A. Caudy, S. M. Hammond, and G. J. Hannon.** 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature **409:**363–366.
5. **Billy, E., V. Brondani, H. Zhang, U. Muller, and W. Filipowicz.** 2001. Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. Proc. Natl. Acad. Sci. USA **98:**14428–14433.
6. **Boutet, S., F. Vazquez, J. Liu, C. Beclin, M. Fagard, A. Gratias, J. B. Morel, P. Crete, X. Chen, and H. Vaucheret.** 2003. Arabidopsis HEN1. A genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. Curr. Biol. **13:**843–848.
7. **Bozzetti, M. P., S. Massari, P. Finelli, F. Meggio, L. A. Pinna, B. Boldyreff, O. G. Issinger, G. Palumbo, C. Ciriaco, S. Bonaccorsi, et al.** 1995. The Ste locus, a component of the parasitic cry-Ste system of Drosophila melanogaster, encodes a protein that forms crystals in primary spermatocytes and mimics properties of the beta subunit of casein kinase 2. Proc. Natl. Acad. Sci. USA **92:**6067–6071.
8. **Djikeng, A., H. Shi, C. Tschudi, and E. Ullu.** 2001. RNA interference in Trypanosoma brucei: cloning of small interfering RNAs provides evidence for retroposon-derived 24–26-nucleotide RNAs. RNA **7:**1522–1530.
9. **Elbashir, S. M., W. Lendeckel, and T. Tuschl.** 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. **15:**188–200.
10. **Elbashir, S. M., J. Martinez, A. Patkaniowska, W. Lendeckel, and T. Tuschl.** 2001. Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J. **20:**6877–6888.
11. **Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello.** 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature **391:**806–811.
12. **Fraser, A. G., R. S. Kamath, P. Zipperlen, M. Martinez-Campos, M. Sohrmann, and J. Ahringer.** 2000. Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. Nature **408:**325–330.
13. **Giordano, E., R. Rendina, I. Peluso, and M. Furia.** 2002. RNAi triggered by symmetrically transcribed transgenes in Drosophila melanogaster. Genetics **160:**637–648.
14. **Grishok, A., A. E. Pasquinelli, D. Conte, N. Li, S. Parrish, I. Ha, D. L. Baillie, A. Fire, G. Ruvkun, and C. C. Mello.** 2001. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell **106:**23–34.
15. **Hall, I. M., G. D. Shankaranarayana, K. Noma, N. Ayoub, A. Cohen, and S. I. Grewal.** 2002. Establishment and maintenance of a heterochromatin domain. Science **297:**2232–2237.
16. **Hamilton, A., O. Voinnet, L. Chappell, and D. Baulcombe.** 2002. Two classes of short interfering RNA in RNA silencing. EMBO J. **21:**4671–4679.
17. **Hammond, S. M., E. Bernstein, D. Beach, and G. J. Hannon.** 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature **404:**293–296.
18. **Hammond, S. M., S. Boettcher, A. A. Caudy, R. Kobayashi, and G. J. Hannon.** 2001. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science **293:**1146–1150.
19. **Hutvagner, G., J. McLachlan, A. E. Pasquinelli, E. Balint, T. Tuschl, and P. D. Zamore.** 2001. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science **293:**834–838.
20. **Hutvagner, G., and P. D. Zamore.** 2002. A microRNA in a multiple-turnover RNAi enzyme complex. Science **297:**2056–2060.
21. **Ishizuka, A., M. C. Siomi, and H. Siomi.** 2002. A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. Genes Dev. **16:**2497–2508.
22. **Kamath, R. S., A. G. Fraser, Y. Dong, G. Poulin, R. Durbin, M. Gotta, A. Kanapin, N. Le Bot, S. Moreno, M. Sohrmann, D. P. Welchman, P. Zipperlen, and J. Ahringer.** 2003. Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature **421:**231–237.
23. **Kennerdell, J. R., S. Yamaguchi, and R. W. Carthew.** 2002. RNAi is activated during Drosophila oocyte maturation in a manner dependent on aubergine and spindle-E. Genes Dev. **16:**1884–1889.
24. **Ketting, R. F., T. H. Haverkamp, H. G. van Luenen, and R. H. Plasterk.** 1999. Mut-7 of C. elegans, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell **99:**133–141.
25. **Lam, G., and C. S. Thummel.** 2000. Inducible expression of double-stranded RNA directs specific genetic interference in Drosophila. Curr. Biol. **10:**957–963.
26. **Livak, K.** 1990. Detailed structure of the Drosophila melanogaster Stellate genes and their transcripts. Genetics **124:**303–316.
27. **Livak, K. J.** 1984. Organization and mapping of a sequence on the Drosophila melanogaster X and Y chromosomes that is transcribed during spermatogenesis. Genetics **107:**611–634.
28. **Llave, C., Z. Xie, K. D. Kasschau, and J. C. Carrington.** 2002. Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science **297:**2053–2056.
29. **Martinez, J., A. Patkaniowska, H. Urlaub, R. Luhrmann, and T. Tuschl.** 2002. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell **110:**563–574.
30. **Mette, M. F., W. Aufsatz, J. van Der Winden, M. A. Matzke, and A. J. Matzke.** 2000. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J. **19:**5194–5201.
31. **Mette, M. F., A. J. Matzke, and M. A. Matzke.** 2001. Resistance of RNA-mediated TGS to HC-Pro, a viral suppressor of PTGS, suggests alternative pathways for dsRNA processing. Curr. Biol. **11:**1119–1123.
32. **Mourelatos, Z., J. Dostie, S. Paushkin, A. Sharma, B. Charroux, L. Abel, J. Rappsilber, M. Mann, and G. Dreyfuss.** 2002. miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev. **16:**720–728.
33. **Nykanen, A., B. Haley, and P. D. Zamore.** 2001. ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell **107:**309–321.
34. **Pal-Bhadra, M., U. Bhadra, and J. A. Birchler.** 2002. RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in Drosophila. Mol. Cell **9:**315–327.
35. **Palumbo, G., S. Bonaccorsi, L. G. Robbins, and S. Pimpinelli.** 1994. Genetic analysis of Stellate elements of Drosophila melanogaster. Genetics **138:**1181–1197.
36. **Papaefthimiou, I., A. Hamilton, M. Denti, D. Baulcombe, M. Tsagris, and M. Tabler.** 2001. Replicating potato spindle tuber viroid RNA is accompanied by short RNA fragments that are characteristic of post-transcriptional gene silencing. Nucleic Acids Res. **29:**2395–2400.
37. **Parrish, S., and A. Fire.** 2001. Distinct roles for RDE-1 and RDE-4 during RNA interference in Caenorhabditis elegans. RNA **7:**1397–1402.
38. **Reinhart, B. J., and D. P. Bartel.** 2002. Small RNAs correspond to centromere heterochromatic repeats. Science **297:**1831.
39. **Rubin, G. M., and A. C. Spradling.** 1982. Genetic transformation of Drosophila with transposable element vectors. Science **218:**348–353.
40. **Schmidt, A., G. Palumbo, M. P. Bozzetti, P. Tritto, S. Pimpinelli, and U. Schafer.** 1999. Genetic and molecular characterization of sting, a gene involved in crystal formation and meiotic drive in the male germ line of Drosophila melanogaster. Genetics **151:**749–760.
41. **Schramke, V., and R. Allshire.** 2003. Hairpin RNAs and retrotransposon

LTRs effect RNAi and chromatin-based gene silencing. Science **301:**1069–1074.
42. **Shevelyov, Y. Y.** 1992. Copies of a Stellate gene variant are located in the X heterochromatin of Drosophila melanogaster and are probably expressed. Genetics **132:**1033–1037.
43. **Shi, H., A. Djikeng, T. Mark, E. Wirtz, C. Tschudi, and E. Ullu.** 2000. Genetic interference in Trypanosoma brucei by heritable and inducible double-stranded RNA. RNA **6:**1069–1076.
44. **Stapleton, W., S. Das, and B. D. McKee.** 2001. A role of the Drosophila homeless gene in repression of Stellate in male meiosis. Chromosoma **110:** 228–240.
45. **Tabara, H., M. Sarkissian, W. G. Kelly, J. Fleenor, A. Grishok, L. Timmons, A. Fire, and C. C. Mello.** 1999. The rde-1 gene, RNA interference, and transposon silencing in C. elegans. Cell **99:**123–132.
46. **Tang, G., B. J. Reinhart, D. P. Bartel, and P. D. Zamore.** 2003. A biochemical framework for RNA silencing in plants. Genes Dev. **17:**49–63.
47. **Thummel, C., A. Boulet, and H. Lipshitz.** 1988. Vectors for Drosophila P-element-mediated transformation and tissue culture transfection. Gene **74:**445–456.
48. **Tijsterman, M., R. F. Ketting, K. L. Okihara, T. Sijen, and R. H. Plasterk.** 2002. RNA helicase MUT-14-dependent gene silencing triggered in C. elegans by short antisense RNAs. Science **295:**694–697.
49. **Tuschl, T., P. D. Zamore, R. Lehmann, D. P. Bartel, and P. A. Sharp.** 1999. Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev. **13:**3191–3197.
50. **Verdel, A., S. Jia, S. Gerber, T. Sugiyama, S. Gygi, S. I. Grewal, and D. Moazed.** 2004. RNAi-mediated targeting of heterochromatin by the RITS complex. Science **303:**672–676.
51. **Volpe, T. A., C. Kidner, I. M. Hall, G. Teng, S. I. Grewal, and R. A. Martienssen.** 2002. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science **297:**1833–1837.
52. **Wilkie, G. S., A. W. Shermoen, P. H. O'Farrell, and I. Davis.** 1999. Transcribed genes are localized according to chromosomal position within polarized Drosophila embryonic nuclei. Curr. Biol. **9:**1263–1266.
53. **Wu-Scharf, D., B. Jeong, C. Zhang, and H. Cerutti.** 2000. Transgene and transposon silencing in Chlamydomonas reinhardtii by a DEAH-Box RNA helicase. Science **290:**1159–1163.
54. **Zamore, P. D., T. Tuschl, P. A. Sharp, and D. P. Bartel.** 2000. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell **101:**25–33.
55. **Zhang, H., F. A. Kolb, V. Brondani, E. Billy, and W. Filipowicz.** 2002. Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. EMBO J. **21:**5875–5885.
56. **Zilberman, D., X. Cao, and S. E. Jacobsen.** 2003. ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. Science **299:**716–719.