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Peculiarities of piRNA-mediated post-transcriptional silencing of *Stellate* repeats in testes of *Drosophila melanogaster*

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ABSTRACT

Silencing of *Stellate* genes in *Drosophila melanogaster* testes is caused by antisense piRNAs produced as a result of transcription of homologous *Suppressor of Stellate (Su(Ste))* repeats. Mechanism of piRNA-dependent *Stellate* repression remains poorly understood. Here, we show that deletion of *Su(Ste)* suppressors causes accumulation of spliced, but not nonspliced *Stellate* transcripts both in the nucleus and cytoplasm, revealing post-transcriptional degradation of *Stellate* RNA as the predominant mechanism of silencing. We found a significant amount of *Su(Ste)* piRNAs and piRNA-interacting protein Aubergine (Aub) in the nuclear fraction. Immunostaining of isolated nuclei revealed co-localization of a portion of cellular Aub with the nuclear lamina. We suggest that the piRNA–Aub complex is potentially able to perform *Stellate* silencing in the cell nucleus. Also, we revealed that the level of the Stellate protein in *Su(Ste)*-deficient testes is increased much more dramatically than the *Stellate* mRNA level. Similarly, *Su(Ste)* repeats deletion exerts an insignificant effect on mRNA abundance of the *Ste-lacZ* reporter, but causes a drastic increase of β-gal activity. In cell culture, exogenous *Su(Ste)* dsRNA dramatically decreases β-gal activity of *hsp70-Ste-lacZ* construct, but not its mRNA level. We suggest that piRNAs, similarly to siRNAs, degrade only unmasked transcripts, which are accessible for translation.

INTRODUCTION

Short RNA molecules are implicated in the regulation of gene expression, defence against viruses and transposable elements and maintenance of genome integrity (1–4). To date, three main classes of repressive short RNA molecules are known in animals: short-interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI interacting RNAs (piRNAs). siRNAs and miRNAs are bound by proteins related to the Ago clade of the Argonaute family and guide them to induce silencing of complementary targets by mRNA degradation, repression of transcription or translation (5,6). piRNAs are bound by the PIWI clade of Argonaute family proteins and differ from miRNAs and siRNAs in length and in the mechanisms of biogenesis and functioning (7–9). The evolutionarily conserved role of piRNAs consists in the regulation of transposable elements and genomic repeats in the germline (7–9). PIWI clade proteins have endonuclease activity and are able to degrade mRNAs of repetitive elements (10–12). *Drosophila* piRNAs can also lead to chromatin silencing of retroelements in ovaries (13). However, the current data poorly elucidate the mechanisms of the repression mediated by piRNAs as compared to those for siRNAs and miRNAs.

Before the acceptance of the 'piRNA' term, Aravin and colleagues (14,15) described in testes of *Drosophila* a short RNA species produced by the Y-chromosome linked *Su(Ste)* (*Suppressor of Stellate*) repeats. The authors revealed that *Su(Ste)* short RNAs are longer than siRNAs and perform silencing of *Su(Ste)* homologous *Stellate* tandem repeated genes located on the X chromosome, which is assisted by the PIWI related Aubergine (Aub) protein. Deletion of the *Su(Ste)* repeats or a mutation in the *aub* gene leads to the elimination of *Su(Ste)* piRNAs and a significant increase of *Stellate* mRNA abundance in testes (14,15). *Stellate* mRNA overexpression causes a drastic accumulation of Stellate protein in crystals, resulting in the disturbance of spermatogenesis and male sterility (16). Recently, it was shown that the Aub protein–*Su(Ste)*–piRNA complex is able to degrade *Stellate* mRNA *in vitro* (11). However, it remains

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unknown whether *Su(Ste)* piRNAs suppress expression of *Stellate* genes by degradation of mRNA or by repression of transcription. Here, we show that the predominant mechanism of *Stellate* genes silencing is post-transcriptional degradation of mRNA, which occurs both in the nucleus and in the cytoplasm. Our results also suggest that the piRNA dependent machinery is able to degrade only a pool of unmasked transcripts, which are accessible for translation.

MATERIALS AND METHODS

Drosophila strains

To produce males carrying the cry^1Y chromosome (deletion of the bulk of *Su(Ste)* repeats on the Y chromosome), $Df(1)w^{67c23(2)}y$ females were crossed to $X/cry^1B^sYy^+$ males, described in Palumbo *et al.* (17). As a control we used $Df(1)w^{67c23(2)}y$, designated as wild type. Strain bearing the $aub^{sting\text{-}1}$ was $y^1ac^1sc^1w^1$ Ste^+;P{lacW}$aub^{sting\text{-}1}$/Cy (18). P-element transformed flies carrying the *Ste703-lacZ* construct (contains the complete *Stellate* promoter and 141 nt of transcribed *Stellate* sequence) were kindly provided by A. A. Aravin. *Ste134-lacZ* transgenic construct containing a shortened (101 nt) *Stellate* promoter and 33 nt of transcribed *Stellate* sequence was described previously (14). All the compared strains carry the same X chromosome.

RT-PCR analysis

Total RNA was isolated from dissected testes or cell culture using Trizol reagent (Invitrogen). The first strand of cDNA was synthesized using the SuperScript II reverse transcriptase (Gibco) and oligo(dT) primer according to a standard procedure. cDNAs were analyzed by real-time quantitative PCR (Chromo4, Bio-Rad) using SYBR Green for detection of *Stellate* cDNA or by semiquantative PCR with αP^{32} dATP for detection of *Ste-lacZ* cDNA. For PCR the following primers were used: 5′-AAGTCTGATACACAGCTGGACGGAGCG-3′ (spl), 5′-GTAATTCTCCGAATATAGTC-3′ (non-spl), 5′-CGCTTGCACTTGCAGTACCTAG-3′ (non-spl2), 5′-CCTGACCAATATTCCGATATTCTTTGGC-3′ (euSte), 5′-GGGTCGTCCAGGGGCGATC-3′ (hetSte2) and 5′-CGATTTGAGTTGCATCAAGGCTTTCA-3′ (hetSte); primers spl/euSte (eu sum), non-spl2/euSte (eu non) were used for amplification of a total of and of nonspliced euchromatic *Stellate* cDNAs, respectively (although the eu sum pair of primers amplifies both spliced and non-spliced PCR products it can be used for detection of spliced transcripts in real-time analysis because its quantity is about 5-fold bigger than quantity of nonspliced transcripts); primers spl/hetSte2 (het sum), non-spl/hetSte (het non) used for amplification of a total of and of nonspliced heterochromatic *Stellate* cDNAs, respectively (for *Stellate* sequences see Supplementary Data); primer specificity was checked by PCR analysis using plasmids carrying eu [plasmid pSX1.3 kindly provided by K.J. Livak (19)] or heterochromatic *Stellate* genes (20) or *Su(Ste)* repeat (Gen Bank accession number Z11735). Other primers were used: 5′-CGGCATCTAAGAAGTGATACTCCCAAAA-3′ (Adh-d3) and 5′-TGAGTGTGCATCGAATCAGCCTTATT-3′ (Adh-r3) for the *Adh* gene (used as a loading control for quantitative real-time RT-PCR of *Stellate* cDNA); 5′-CAGGCCCAAGATCGTGAAG-3′ (rp49d) and 5′-TGAGAACGCAGGCGACC-3′ (rp49r) for the *rp49* gene (used as a loading control for semiquantative RT-PCR of *Ste-lacZ* cDNA); 5′-GTGGTTATGCCGATCGCGT-3′ (lacZ1) and 5′-ATATCGGTGGCCGTGGTGT-3′ (lacZ2) for *lacZ* (used for amplification of *Ste-lacZ* cDNA obtained from testes RNA); 5′-GGCGAGGAGCTGTTCACC-3′ (GFP1) and 5′-TGCTCAGGTAGTGGTTGTCG-3′ (GFP2) for *GFP*; 5′-GGCATGATTCACGCCCGATACAT-3′ (Ste) and 5′-CGATTAAGTTGGGTAACGCCAG-3′ (lacZ3) for *Ste-lacZ*, lacZ1 and 5′-ACCGCCAAGACTGTTACCCAT-3′ (lacZ4) for *lacZ* (used for amplification of *Ste-lacZ* or *lacZ* cDNA obtained from cell culture RNA).

X-gal staining and β-gal activity assay

X-gal staining and β-gal activity assays were performed according to protocols described previously (14,15). Five to 10 testes dissected from 1–3-day- old males or cell culture were used for β-gal activity assay. To normalize measurements of β-gal activity we equalized the total testes protein evaluated by the Bio-Rad protein assay kit or analyzed an equal quantity of the cell culture cells.

Separation of nuclear and cytoplasmic fractions

Testes were manually dissected with needles in 1× PBS solution (2 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.3), precipitated, transferred to lysis buffer [350 mM sucrose, 15 mM HEPES (pH 7.6), 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 1 mM DTT, Protease inhibitor cocktail (Roche), 0.5 U RNasin Plus (Promega)] and homogenized by Dounce homogenizer on ice. Lysate was filtered through Mirocloth membrane (Calbiochem) and spun at 1800 *g* for 10 min at 4°C. Pellet 1 and supernatant 1 fractions were separated from each other. Pellet 1 (nuclei) was washed by lysis buffer producing pellet 2 and supernatant 2. Pellet 2 was pipetted in resuspension buffer [290 mM sucrose, 5 mM Tris–HCl (pH 7.4), 1.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, Triton X-100 0.04%, Protease inhibitor cocktail (Roche), 0.5 U RNasin Plus (Promega)], incubated at rocking for 12 min at 4°C for removing nuclear membranes according to (21) and spun at 200 *g* for 6 min at 4°C. Pellet 3 was considered as nuclear fraction and resuspended in lysis buffer. Supernatants 1, 2 and 3 were pooled and considered as cytoplasmic fraction. One-fifth of the nuclear and cytoplasmic fractions was used for western analysis and the remaining material was used for northern analysis.

RNA isolation and detection by northern blot

RNA was isolated from nuclear and cytoplasmic fractions using Trizol reagent (Invitrogen). Total RNA was quantified by absorbance at 260 nm, and 0.4–8 μg of total RNA was resolved by 20% denaturing polyacrylamide/urea gel electrophoresis (Mini-PROTEAN Tetra Electrophoresis System, Bio-Rad) in 1× TBE. 5′-^{33}P-radiolabeled RNA

oligonucleotides were used as size markers. After electrophoresis, the polyacrylamide gel was transferred to Hybond N + membrane (Amercham) in 0.5×TBE by electrophoretic transfer (Mini Trans-Blot Cell, Bio-Rad) at 250 mA for 1 h. The RNA was cross-linked to the membrane by UV irradiation (1200 μjoules/cm; Biolink DNA Crosslinker, Biometra). Membrane was prehybridized in Church buffer [0.5 M phosphate buffer (pH 7.5), 1 mM EDTA, 7% SDS] for 2 h at 37°C for oligonucleotide probes and in prehybridization buffer [50 mM phosphate buffer (pH 7.5), 0.5 M NaCl, 0.1% Ficol400, 0.1% polyvinilpyrolidone, 0.1% pyrophosphate, 50 μg/ml heparin, 25 mM EDTA, 1% sarcosyl, 150 μg/ml denaturated DNA] for 2 h at 50°C for riboprobes. Fifteen picomoles of DNA probe (Syntol, Moscow, Russia) was 5′-^{32}P-radiolabeled (81 μCi γ-^{32}P-dATP) with polynucleotide kinase (NEB) and purified using Sephadex G-25 spin column (Bio-Rad). ^{32}P-labeled riboprobe was transcribed by T7 RNA polymerase (Ambion) with 27 μCi α-^{32}P-dATP using the Su(Ste) fragment as a template, producing an antisense RNA probe for hybridization. After synthesis, the labeled RNA was partially hydrolyzed during 1-h incubation at 60°C in the presence of 80 mM $NaHCO_3$, 160 mM Na_2CO_3. The ^{32}P-radiolabeled probes were hybridized for 16–20 h in Church buffer or in prehybridization buffer with 25% formamide for oligonucleotide probe or riboprobe, respectively. After hybridization, membranes were sequentially washed with 2× SSC/0.1% (w/v) SDS, 1× SSC/0.1% (w/v) SDS and 0.5× SSC/0.1% (w/v) SDS for 10 min and analyzed by phosphorimager (Storm, Amersham) using Image Quant computational tool. To strip probes, membranes were incubated in Church buffer for 2 h at 70°C and then re-exposed to confirm probe removal. The following DNA probes were used: 5′-GGGTATGAACCCAGTAGCTTAA-3′ (mt tRNA M), 5′-AGATTAAGAGTCTCATGCTCTA-3′ (tRNA K), 5′-TCGGGCTTGTTCTACGACGATG-3′ (Su(Ste)-4 piRNA).

Western blot analysis

Quantity of protein lysates used for western analysis was equalized by the Bio-Rad protein assay kit. To estimate the efficiency of nuclear and cytoplasmic fractions separation mouse monoclonal antibodies to lamin DmO ADL67.10 (Developmental Studies Hybridoma Bank) and rabbit monoclonal antibodies to γ-tubulin (Sigma) and rabbit polyclonal antibodies to calnexin were used at 1:1000, 1:7000 and 1:2000 dilution, respectively. To detect the Aub protein rabbit polyclonal antibodies to N-terminal 16 AA of Aub kindly provided by G. Hannon were used at 1:2000 dilution. To detect Stellate protein in testis extracts anti-Stellate mouse polyclonal antibodies (Egorova K., manuscript in preparation) were used at 1:3000 dilution. To estimate loading mouse monoclonal antibodies to beta-actin ab8224 (Abcam, Cambridge, UK) were used at 1:800 dilution. Alkaline-phosphatase-conjugated anti-mouse or anti-rabbit IgG (whole molecules) (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) were used as a secondary reagent. Samples were resolved by electrophoresis in 12% or 8% PAGE/SDS and blotted onto the PVDF membrane Immobilon-P (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). Blots were developed using the Immun-Star AP detection system (Bio-Rad Laboratories, Hercules, CA, USA) according to the recommendations of the manufacturer.

RNAi in cell culture

dsRNA was transcribed by T7 RNA polymerase (Ambion) using the Su(Ste), Ste, lacZ or GFP PCR products with two opposite T7 promoters as templates. To obtain dsRNA duplexes, mixtures were heated to 60°C and slowly cooled in water bath. *Drosophila* S2 cells (5×10^6) were transfected by the Ca transformation method (22) with *hsp70-GFP* (pHSBJ) (15 μg), *hsp70-Ste-lacZ* (pCaSpeR, construct contain 585 nt of transcribed *Stellate* sequence) (15 μg) or *hsp70-lacZ* (pCaSpeR) (15 μg) and one of the dsRNAs (*GFP*, *Su(Ste)*, *Ste* or *lacZ*) 300–600 nt in length (1.5 μg). Three days after transfection, heat-shock procedure of cells was performed for 20 min at 37°C. Four hours later, β-gal activity assays and RNA isolation for RT-PCR were done.

Immunocytochemistry of testes and nuclei

Testes were dissected in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS at room temperature for 1 h. Testes were incubated two times for 30 min in PBTD solution (PBS + 0.1% Tween-20 + 0.3% Triton X-100 + 0.3% sodium deoxycholate), rinsed three times in PBT (PBS + 0.1% Tween-20) for 5 min and blocked in 3% goat antiserum in PBT plus 0.3% Triton X-100 for 1–2 h at room temperature. Testes were incubated with primary antibodies diluted 1:500 in PBT plus 0.3% Triton X-100 for 1 h at room temperature. We used rabbit anti-Aub polyclonal antibodies to N-terminal 16 AA of Aub kindly provided by G. Hannon and mouse monoclonal antibodies to lamin DmO ADL67.10 (Developmental Studies Hybridoma Bank). Then testes were rinsed three times for 15 min in PBT, incubated with secondary antibodies for 3 h at room temperature or at 4°C overnight, stained with DAPI for 15 min in PBT and washed for 15 min in PBT again. We used Alexa Fluor 647 conjugated anti-rabbit antibody and Alexa Fluor 488 conjugated anti-mouse antibody (Invitrogen). All secondary antibodies were diluted 1:1000. Then PBT was replaced by SlowFade Gold antifade reagent (Invitrogen) and testes were transferred to glass slides and analyzed by a confocal microscope (Carl Zeiss).

The nuclear fraction was spread on a work surface of microscope slides (SuperFrost Plus Gold, Menzel-Glaser) and incubated for 15 min to allow nuclei to settle down. Nuclear fraction was fixed in 1.8% paraformaldehyde in PBS at room temperature for 15 min, rinsed three times in PBT for 5 min and blocked in 3% goat antiserum in PBT plus 0.3% Triton X-100 for 1–2 h at room temperature. The incubations with antibodies were the same as for testes analysis.

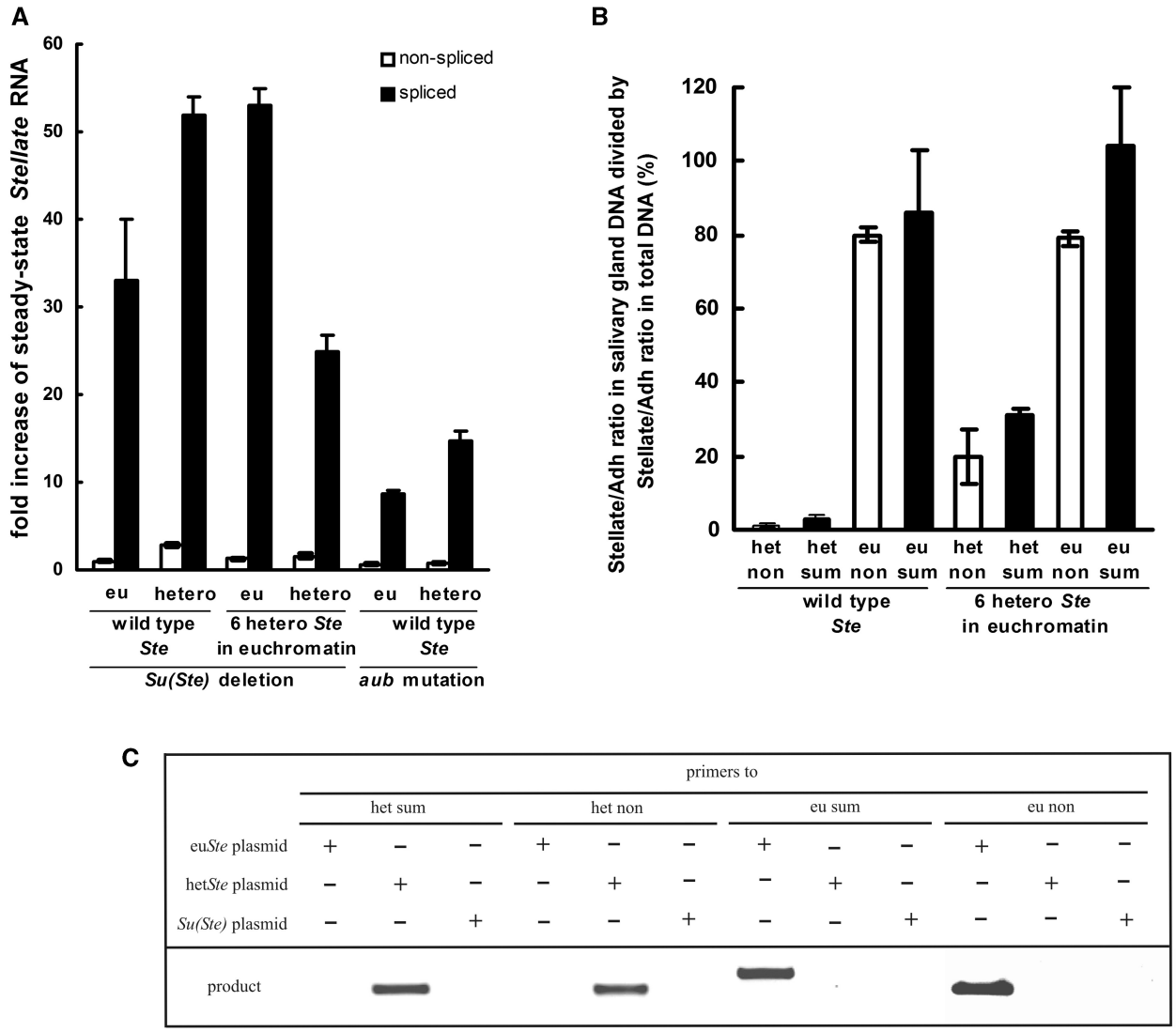

Figure 1. The effect of *Su(Ste)* repeats deletion or *aub* mutation on spliced and non-spliced *Stellate* mRNA abundance in the testes of *Drosophila melanogaster*. (**A**) Deletion of *Su(Ste)* repeats (compared with wild type) and the $aub^{sting\text{-}1}$ (homozygous flies compared with heterozygous ones) lead to an increase of spliced euchromatic (eu) and heterochromatic (hetero) *Stellate* transcripts abundance (dark bars), but not nonspliced transcripts abundance (light bars). Quantitative RT-PCR was done using primers detecting either nonspliced (pairs of primers designated as eu non or het non in the text) or a sum of spliced and nonspliced *Stellate* transcripts (designated as eu sum or het sum). The quantity of spliced transcripts was calculated by subtracting the nonspliced transcript quantity from the sum. We were able to detect euchromatic and heterochromatic *Stellate* transcripts separately, since the 3′ end nucleotides of the primers were complementary to variants of single nucleotide polymorphisms, which distinguish types of *Stellate* genes (see Supplementary Data). The middle four bars show the effect of *Su(Ste)* deletion causing derepression of *Stellate* genes in the fly strain carrying a transgenic construct with six heterochromatic *Stellate* genes in the euchromatin of chromosome 3. (**B**) PCR analysis with plasmids carrying eu- or heterochromatic *Stellate* genes or a *Su(Ste)* repeat confirms primers specificity. (**C**) Verification of localization of detected *Stellate* copies. The diagram shows values, which correspond to the results of dividing the *Stellate*/*Adh* ratio in salivary gland DNA by the *Stellate*/*Adh* ratio in total DNA. This test confirms that het non and het sum or eu non and eu sum primers detect *Stellate* copies located in the hetero- or euchromatin, correspondingly. Using of fly strain with six heterocromatic *Stellate* genes in the euchromatin leads to increasing of the values for primers specific to heterochromatic *Stellate* genes confirming validity of the test.

RESULTS

Expression of *Stellate* repeats is regulated at the post-transcriptional level

To elucidate whether the repression of *Stellate* genes by *Su(Ste)* piRNAs occurs at the transcriptional or post-transcriptional level, we estimated the effect of *Su(Ste)* deletion or the $aub^{sting\text{-}1}$ mutation on the abundance of spliced and nonspliced *Stellate* transcripts in testes by quantitative RT-PCR. Deletion of the *Su(Ste)* locus or $aub^{sting\text{-}1}$ lead to the disappearance of *Su(Ste)* piRNAs (14,15). If *Su(Ste)* piRNAs suppressed transcription of *Stellate* genes, the loss of silencing would cause a comparable increase of spliced and newly transcribed nonspliced transcripts quantity. We found that both *Su(Ste)* deletion and $aub^{sting\text{-}1}$ lead to an insignificant increase of non-spliced transcript amount, while the level of spliced transcripts is dramatically increased (10–50 times) (Figure 1A).

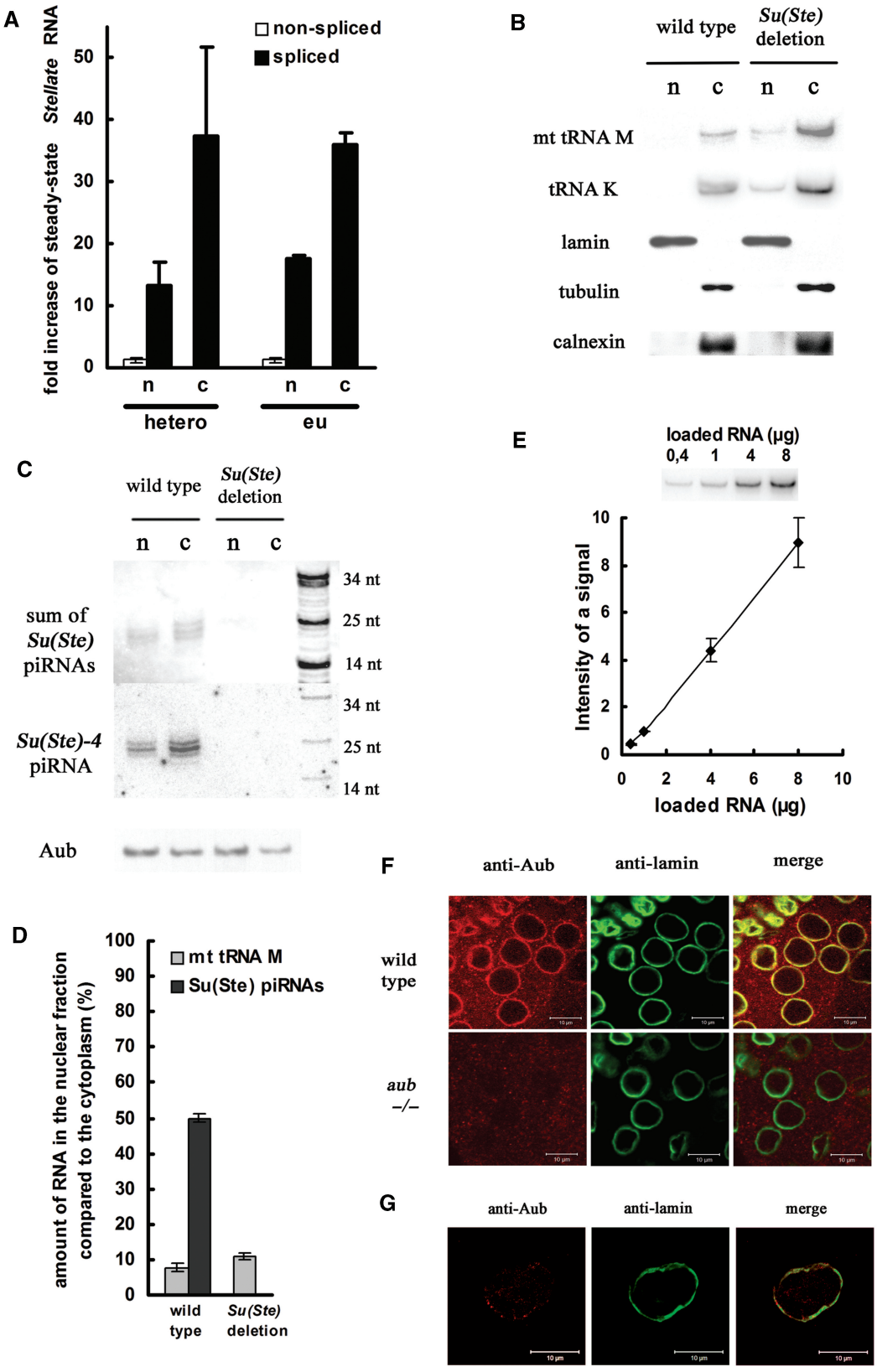

Figure 2. Degradation of *Stellate* mRNA by the piRNA machinery occurs both in the nucleus and the cytoplasm. (**A**) Deletion of *Su(Ste)* repeats leads to an increase of spliced euchromatic (eu) and heterochromatic (hetero) *Stellate* transcripts quantity both in the nuclear (n) and cytoplasmic (c) fractions. (**B**) Estimation of the purity of nuclear and cytoplasmic fractions. Upper rows: northern analysis with probes complementary to mitochondrial methionine tRNA (mt tRNA M) and cytoplasmic lysine tRNA (tRNA K) in nuclear (n) and cytoplasmic (c) fractions. Lower rows: western analysis with antibodies against nuclear lamin, cytoplasmic γ-tubulin and membrane marker calnexin proteins. (**C**) *Su(Ste)* piRNAs and the

Thus, we consider posttranscriptional mRNA degradation as the predominant mechanism of *Stellate* silencing.

The genome of *Drosophila melanogaster* contains two clusters of *Stellate* genes located in the 12D euchromatic region and in the distal X-heterochromatin (17,19,20,23). The designed primers allowed us to separately detect *Stellate* transcripts derived from both clusters, owing to the single nucleotide polymorphisms (19,20) (see Supplementary Data). PCR analysis of plasmids, containing euchromatic or heterochromatic *Stellate* copies or a *Su(Ste)* repeat, confirmed the specificity of primers (Figure 1B). To verify that detected *Stellate* copies have eu- or heterochromatic localization in the genome, we used a well-known approach based on the phenomenon of heterochromatic DNA under-replication in the nuclei of salivary glands (24), where euchromatic DNA undergoes multiple polytenization, while heterochromatic DNA is not replicated. We related quantities of euchromatic or heterochromatic *Stellate* genes to the euchromatic Adh gene using quantitative Real-Time PCR of DNA isolated from whole flies and from larvae salivary glands. We evaluated euSte/Adh and hetSte/Adh ratios for the salivary glands DNA and calculated the percentage to the corresponding values for DNA from whole flies (Figure 1C). It was shown, that amount of heterochromatic *Stellate* PCR products, amplified by two different pairs of primers, is dramatically decreased in salivary gland DNA compared to total DNA. Thus, the used primers are able to discriminate between euchromatic or heterochromatic *Stellate* copies. For both types of *Stellate* genes, *Su(Ste)* deletion and $aub^{sting\text{-}1}$ mutation cause an increase of spliced but not nonspliced transcripts level (Figure 1A). Thus, our data suggests that the chromatin state does not affect the mode of *Stellate* silencing.

Nuclear compartment contributes to piRNA-dependent *Stellate* silencing

It was shown that siRNA-mediated degradation of transcripts may occur in the cytoplasm (25,26) and in the nucleus (27,28), but it is poorly understood where piRNA-mediated degradation takes place. We examined the effect of *Su(Ste)* deletion on *Stellate* transcript abundance in the nuclei and the cytoplasm separately. Nuclear and cytoplasmic fractions were obtained from lysates of *Su(Ste)*-deficient and wild-type testes. Quantitative RT-PCR revealed that *Su(Ste)* deletion leads to 38- and 13-fold increase of spliced heterochromatic *Stellate* mRNA amount in the cytoplasmic and nuclear fraction, respectively (Figure 2A). A similar result was obtained for euchromatic *Stellate* mRNA. The purity of the fractions was evaluated by western analysis using antibodies against lamin and γ-tubulin, which are nuclear and cytoplasmic proteins, respectively (Figure 2B). According to the procedure of fractionation nuclear fraction should be free of nuclear membranes (21). In order to control this, western analysis with an antibody against the endoplasmic reticulum membrane marker calnexin protein was done (Figure 2B). Contamination of the nuclear fraction by the cytoplasm was also estimated by northern analysis with a probe complementary to cytoplasmic lysine tRNA and mitochondrial methionine tRNA (Figure 2B). According to both western and northern analyses, the extent of nuclear fraction contamination by the cytoplasm does not exceed 10% (Figure 2D). Thus, the observed significant accumulation of *Stellate* transcripts in the nuclear fraction can not be explained by cytoplasmic contamination. We conclude that *Stellate* mRNA degradation occurs both in the nucleus and the cytoplasm. This result suggests that the complex of the Aub protein and *Su(Ste)* piRNAs performing silencing of *Stellate* genes (11,14) may be found both in the nuclear and cytoplasmic fractions. Using northern hybridization, we detected *Su(Ste)* piRNAs in both fractions (Figure 2C). We used a riboprobe detecting the sum of *Su(Ste)* piRNAs, or an oligonucleotide probe complementary to the individual *Su(Ste)-4* piRNA, which was shown to be the most abundant among testes piRNAs immunoprecipitated with the Aub protein (11). In the nuclear fraction, *Su(Ste)* piRNAs are only 2-fold less abundant than in the cytoplasm (Figure 2D). Western analysis revealed the presence of the Aub protein also both in the nuclear and cytoplasmic fractions (Figure 2C). We also performed immunostaining to examine the localization of Aub in whole testes (Figure 2F) and isolated nuclei (Figure 2G). In the spermatocytes Aub is detected as a bright perinuclear ring (Figure 2F) representing the so-called nuage structure (29). In the isolated nuclei it was weaker, but a significant signal remained to be co-localized with the lamina (Figure 2G). Taking into account Aub co-localization with the lamina, we suggest that Aub is localized not only in the perinuclear organelle nuage, but also on the inner side of the nuclear membrane. Thus, fractionation experiments and immunostaining indicate that the piRNA–Aub complex is located both in the nucleus and cytoplasm.

Su(Ste) piRNAs affect Stellate protein level much more dramatically than mRNA level

Western assay with anti-Stellate antibodies reveals that Stellate protein level in *Su(Ste)*-deficient testes increases much more dramatically (>200-fold) than the *Stellate* mRNA level (20- to 50-fold) (Figures 1 and 3A). Similar results we obtained using transgenic flies carrying

Aub protein are found both in nuclear and cytoplasmic fractions. Upper row: northern analysis with a riboprobe complementary to a pool of *Su(Ste)* piRNAs. Middle row: northern analysis with a DNA oligonucleotide complementary to a unique *Su(Ste)*-4 piRNA. Lower row: Western analysis with antibodies against Aub. (**D**) Amount (%) of mitochondrial methionine tRNA (from B) and *Su(Ste)* piRNAs (from C) in the nuclear fraction as compared to the cytoplasm. (**E**) Northern analysis confirms the proportionality of the hybridization signal to the amount of loaded RNA (methionine tRNA probe). (**F**) Localization of Aub in *Drosophila* testis. Testes were immunostained with anti-Aub (shown in red) and anti-lamin (shown in green). Specificity of anti-Aub was verified by immunostaining of testes of aub^{HN}/aub^{QC42} (–/–) trans-heterozygous mutant flies. Scale bars: 10 μm. (**G**) Aub co-localizes with lamina in the nuclei. Nuclear fraction was immunostained with anti-Aub and anti-lamin. A dot-like signal of Aub co-localizes with lamina.

Ste-lacZ reporter constructs driven by the *Stellate* promoter (*Ste703-lacZ* or *Ste134-lacZ* constructs contain the whole or 101 nt of *Stellate* promoter and first 141 or 33 nt of transcribed *Stellate* sequences, respectively). Deletion of *Su(Ste)* repeats exerts an insignificant effect on *Ste-lacZ* mRNA abundance (no more than 1.5-fold), but causes 4–7-fold increase of β-gal activity (Figure 3B and C). Slight increase of *Ste-lacZ* expression as compared to the endogenous *Stellate* genes level may be attributed to the structure of *Ste-lacZ* constructs, which contain incomplete *Stellate* sequences.

A fraction of *Ste-lacZ* mRNA is protected from siRNAs in S2 cells

The usage of reporter constructs allowed us to compare the peculiarities of piRNA- and siRNA-mediated repression. We performed dsRNA-mediated knockdown of the *hsp70-Ste-lacZ* construct in S2 cell culture. The *hsp70-Ste-lacZ* plasmid contains the first 585 nt of transcribed *Stellate* sequence fused to *lacZ* driven by the *hsp70* promoter. Cotransfection of the *hsp70-Ste-lacZ* plasmid and *Su(Ste)*, *Ste* or *lacZ* dsRNAs leads to a 7-10-fold decrease of β-galactosidase activity, but no more than 1.5–2-fold decrease of the mRNA level (Figure 3D). This result revealed similarities of piRNA and siRNA effects on the expression of *Ste-lacZ* constructs, resulting in a drastic decrease of protein level without a significant decrease of the abundance of *Ste-lacZ* mRNA. Note that in cell culture dsRNA digestion produces siRNAs, which are incorporated into Ago2 complexes and catalyze degradation of complementary mRNAs, but cannot perform repression of translation (30,31). To estimate the efficiency of mRNA degradation by dsRNA in our system, knockdown of *hsp70-GFP* plasmid by *GFP* dsRNA was done and 10-fold decrease of *GFP* mRNA quantity was observed (Figure 3E). Thus, we conclude that a significant fraction of the *Ste-lacZ* mRNA is not degraded by siRNA-containing effector complexes. To estimate whether the *Stellate* sequence *per se* is responsible for *Ste-lacZ* mRNA protection from degradation, a knockdown of *hsp70-lacZ* plasmid by *lacZ* dsRNA was also done, leading to a 5-fold decrease of *lacZ* mRNA quantity (Figure 3F), that is significantly stronger than the observed effect on the *Ste-lacZ* mRNA (about 1.3-fold decrease). Presumably, *Ste-lacZ* transcripts are protected by putative masking proteins, interacting with specific sequence motifs of *Stellate* RNA. Similarity of the results, which we obtained studying piRNA- and siRNA-dependent *Ste-lacZ* repression, allow us to suggest that only an unprotected fraction of total cytoplasmic *Stellate* RNA in testes is accessible both for piRNA-mediated degradation and translation.

DISCUSSION

Silencing of the testes expressed *Stellate* genes has been shown to be associated with short piRNAs recognized by the PIWI clade protein Aub (11,32). However, it remains unclear whether this mechanism is operated at the transcriptional or post-transcriptional level. Here, we show that deletion of *Su(Ste)* repeats, producing piRNAs, or *aub* mutation causes accumulation of spliced, but not nascent *Stellate* transcripts, revealing post-transcriptional degradation of *Stellate* RNA as the predominant mechanism of silencing. At the same time, the role of piRNAs in the transcriptional silencing of mobile elements in mammals and *Drosophila* has been suggested (9,13,33–35). In *Drosophila* ovaries a mutation decreasing piRNA production results in the opening of chromatin of transposable elements (13). Suggested piRNA-depending chromatin silencing is presumably caused by another PIWI clade protein Piwi (9), that is localized in the nuclei of ovarian cells, but is not expressed in the testes spermatocytes (10,11,36), where Aub-dependent *Stellate* repression occurs (Refs 11 and 15 and in this work). Thus, the piRNA machinery may be involved both in chromatin closing and post-transcriptional silencing.

We found that degradation of *Stellate* mRNA may occur not only in the cytoplasm, but also in the nuclei. The Aub protein, which performs silencing of the *Stellate* genes by *Su(Ste)* piRNAs (11,32), was detected in the cytoplasm and perinuclear structure called nuage in ovarian germinal cells (29,37,38). We found a significant amount of Aub in testis nuclear fraction, which was free of nuclear membranes according to the procedure of isolation (21) and western analysis with an antibody against endoplasmic reticulum membrane marker calnexin. Immunostaining of the testes or nuclei showed co-localization of Aub and lamin, which is an inner nuclear-membrane-associated protein. Patterns of Aub localization are very similar to those detected by previous authors (29,38). Using of antibodies to lamin allowed us to visualize a detectable amount of Aub on the inner side of nuclear membrane in isolated nuclei. We suggest that the piRNA–Aub complex is potentially able to perform *Stellate* silencing in the nucleus and cytoplasm.

Our previous data have shown the accumulation of antisense *Su(Ste)* transcripts in the nuclei of early stages of spermatocyte maturation (15). This observation emphasizes the possibility of antisense piRNA production in the nuclei and its immediate involvement in *Stellate* silencing.

We found that the loss of piRNAs in testes caused by the removal of *Su(Ste)* repeats leads to a significantly more strong increase of the Stellate protein level (more than 200-fold) than *Stellate* mRNA abundance (30–50-fold). The same effect we observed using transgenic *Ste-lacZ* constructs carrying fragments of the *Stellate* gene fused to *lacZ*. In this case, *Su(Ste)* deficiency also causes a drastic upregulation of β-gal activity (4–7-fold) whereas the *Ste-lacZ* transcript abundance increases insignificantly (no more than 1.5-fold). The usage of *Ste-lacZ* constructs allowed us to compare the response of their expression to *Su(Ste)* piRNA *in vivo* and to siRNA in cell culture. Knockdown of *Ste-lacZ* construct by siRNAs in S2 cells causes a drastic decrease of β-gal activity (7–10-fold), but does not significantly affect the amount of *Ste-lacZ* mRNA (about 1.5-fold). Taking into account the much more strong decrease of *GFP* or *lacZ* mRNA levels caused by *GFP* or *lacZ* siRNAs (10- and 5-fold, respectively), we propose that the bulk of *Ste-lacZ* mRNA is associated with putative ubiquitous

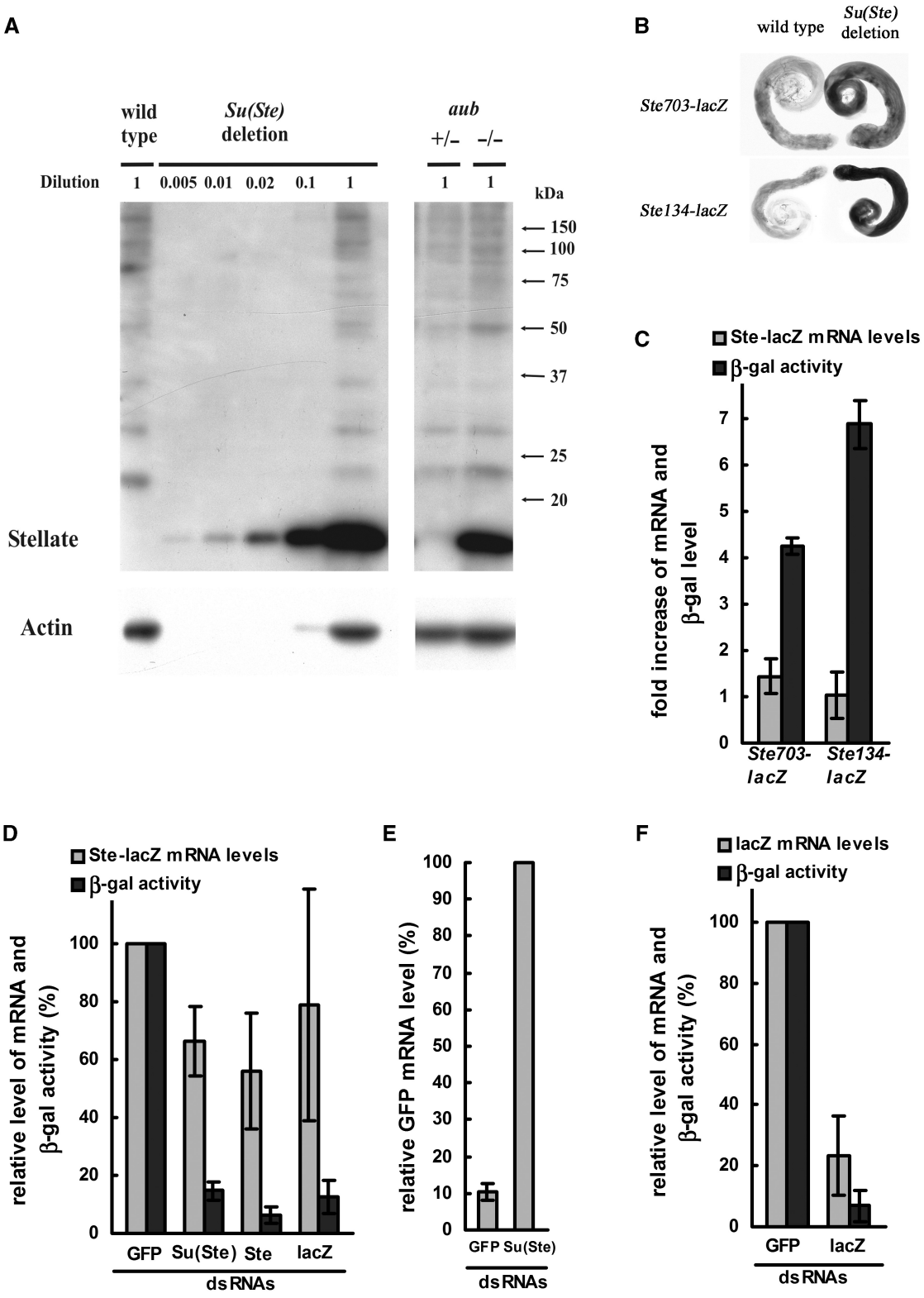

Figure 3. (**A**) Deletion of *Su(Ste)* repeats or $aub^{sting\text{-}1}$ lead to a more than 200-fold increase of the Stellate protein level. Different quantities of lysates from testes of *Su(Ste)*-deficient flies were used for western analysis with antibodies against the Stellate protein and actin. (**B**) X-gal staining of wild-type and *Su(Ste)*-deficient testes, carrying transgenic reporters *Ste703-lacZ* or *Ste134-lacZ*. (**C**) Fold increase of *Ste-lacZ* mRNA level (light bars) and β-gal activity (dark bars) in testes of *Su(Ste)*-deficient males relatively to the wild-type ones. (**D–F**) S2 cell culture was transfected by plasmids encoding a *Ste-lacZ* reporter construct (D), or the *GFP* gene (E) or a *lacZ* reporter construct (F) driven by a heat-shock promoter and one of dsRNAs [*GFP*, *Su(Ste)*, *Ste* or *lacZ* in (D) and *GFP* or *Su(Ste)* in (E) and (F)]. Decrease of abundance of mRNA (D–F) and β-gal activity (D and F) owing to transfection by homologous dsRNAs is shown.

masking proteins, interacting with specific sequence motifs of *Stellate* RNA and preventing siRNA-mediated degradation. These data suggest that piRNAs in testes, similarly to siRNAs in S2 cells, cause degradation of translation accessible unmasked transcripts, rather than repression of translation.

The proposed mode of piRNA-mediated posttranscriptional silencing could occur in the course of silencing of transposable elements. It was shown that the disruption of the piRNA machinery leads to a drastic increase of transposition rate of the mdg1 retrotransposon, but a slight increase of mdg1 transcript level (39). We suppose that the piRNA system may be aimed predominantly at elimination of translation accessible mRNAs, preventing the synthesis of reverse transcriptases, transposases and other transposon-encoded proteins.

Our results show that piRNAs-mediated post-transcriptional silencing of *Stellate* genes takes place both in the nucleus and in the cytoplasm and *Su(Ste)* piRNAs slicing activity may be preferentially directed to the cytoplasmic transcripts, which are accessible for translation. These peculiarities of piRNA action seem to be similar to those described for siRNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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