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Note

Transcription of the 1.688 Satellite DNA Family Is Under the Control of RNA Interference Machinery in *Drosophila melanogaster* Ovaries

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ABSTRACT

Here we show that RNA interference (RNAi) machinery operates in *Drosophila melanogaster* 1.688 satellite transcription. Mutation in the *spn-E* gene, known to be involved in RNAi in the oocytes, causes an increase of satellite transcript abundance. Transcripts of both strands of 1.688 satellite repeats in germinal tissues were detected. The strength of the effects of the *spn-E* mutation differs for 1.688 satellite DNA subfamilies and is more pronounced for autosomal pericentromeric satellites compared to the X-linked centromeric ones. The *spn-E*^{1} mutation causes an increase of the H3-AcK9 mark and TAF1 (a component of the polymerase II transcriptional complex) occupancy in the chromatin of autosomal pericentromeric repeats. Thus, we revealed that RNAi operates in ovaries to maintain the silenced state of centromeric and pericentromeric 1.688 repeats.

RNA interference (RNAi) has been implicated in recognizing repetitive DNA elements as preferential targets for heterochromatin assembly (Volpe *et al.* 2002; Birchler *et al.* 2004; Lippman *et al.* 2004; Verdel *et al.* 2004; Kavi *et al.* 2005). The mechanism of transcriptional silencing of centromeric repeats in *Schizosaccharomyces pombe* has been extensively studied. The *S. pombe* centromeric repeats are transcribed in both directions, producing double-stranded RNAs (dsRNAs), which are processed into small interfering RNAs (siRNAs) by the endonuclease Dicer. RNA-induced transcriptional silencing (RITS) complexes carrying siRNAs are thought to be associated with nascent centromeric transcripts, to recruit H3-K9 methyltransferase, and to provide formation of the self-sustaining closed chromatin state (Verdel *et al.* 2004; Buhler *et al.* 2006; Sugiyama *et al.* 2007). The heterochromatic state of *S. pombe* centromeric repeats is needed for proper centromere functioning, including cohesin binding (Partridge *et al.* 2002). Centromeric satellite repeats in Arabidopsis and mouse are also transcribed bidirectionally, ensuring formation of dsRNAs and siRNA generation (Martens *et al.* 2005; May *et al.* 2005; Lee *et al.* 2006).

siRNAs corresponding to 1.688 satellite have been detected in the Drosophila siRNA library (Aravin *et al.* 2003). To address whether satellite DNA transcription is under RNAi control, we analyzed the effects of *spn-E*^{1}, *spn-E*$^{hls\text{-}03987}$, and *aub*^{QC42} mutations on the transcriptional status of the 1.688 satellite DNA. The *spn-E* gene, encoding a DExD-box RNA helicase (Gillespie and Berg 1995), as well as the *aub* gene, a member of the Piwi family, are involved in dsRNA-triggered RNAi in embryos (Kennerdell *et al.* 2002), in transcriptional silencing of transgenes (Pal-Bhadra *et al.* 2004), and in the control of Drosophila retrotransposon transcript abundance in the germline (Aravin *et al.* 2001; Savitsky *et al.* 2006; Vagin *et al.* 2006).

In the *Drosophila melanogaster* genome, each centromeric region seems to contain different sets of satellite DNA sequences (Abad *et al.* 1992, 2000; Lohe *et al.* 1993; Sun *et al.* 1997; Agudo *et al.* 1999; Lamb and Birchler 2003). The *X* chromosome contains a large block of complex 1.688 satellite DNA (359-bp repeat unit), located in the centromeric region and in the adjacent

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pericentromeric heterochromatin (Lohe *et al.* 1993; Abad *et al.* 2000) (Figure 1A), and large autosomes contain arrays of different subfamilies of 1.688 satellite DNA in the pericentromeric heterochromatin. The 260-bp arrays are located at 2L heterochromatin (Abad *et al.* 2000) (Figure 1A), while the 353- and 356-bp arrays are attributed to distal and proximal regions of 3L heterochromatin, respectively (Losada and Villasante 1996) (Figure 1A). The 361-bp arrays are located in a region adjacent to the 356-bp arrays (A. Villasante, unpublished results) (Figure 1A). The transient presence of the kinetochore-specific protein BubRI in all heterochromatic regions containing the 1.688 satellite has been shown by immunostaining (Abad *et al.* 2000). BubR1 is required for the spindle checkpoint function and for chromosome alignment (Ditchfield *et al.* 2003), suggesting a role of 1.688 satellites in chromosome congression. Short tandem arrays (two to four copies) of sequences homologous to the 1.688 satellite are dispersed throughout the euchromatin of the *X* chromosome. These 1.688 satellite-related sequences are often situated in the vicinity of genes and their requirement for some sex-chromosome-specific functions has been discussed (Waring and Pollack 1987; DiBartolomeis *et al.* 1992; Losada and Villasante 1996).

Here we show that the 1.688 satellite DNA is transcribed bidirectionally in germinal tissues of *D. melanogaster*. Mutations in the *spn-E* and *aub* genes resulted in the increase of satellite transcript abundance in ovaries and the *spn-E*^{1} mutation causes the accumulation of the histone H3 acetylated at lysine 9 (H3-AcK9) mark and transcription factor TAF1 in satellite chromatin.

MATERIALS AND METHODS

Drosophila strains: The strain bearing the *spindle-E* (*spn-E*^{1}) mutation was *ru*^{1} *st*^{1} *spn-E*^{1} *e*^{1} *ca*^{1}*/TM3, Sb*^{1} *e*^{s} (bearing a point mutation in the helicase domain); the *aub* mutant was aub^{QC42}/CyO.

RT–PCR analyses: RT–PCR was done according to described procedure (Aravin *et al.* 2001) using pairs of primers corresponding to 1.688 satellite subfamilies: 361 bp, 5′-TCA ACGATGTATGACATTCC-3′ (R, right) and 5′-TGAGCTCG TAATAAAATTTCC-3′ (L, left); 260 bp, 5′-ATGAAACTGTGT TCAACAAT-3′ (R) and 5′-TGGAAATTTAATTACGAGCT-3′ (L); 359 bp, 5′-TATTCTTACATCTATGTGACC-3′ (R) and 5′-GTTTTGAGCAGCTAATTACC-3′ (L). As a loading control, we used the primers for rp49 (5′-ATGACCATCCGCCCAGCA TAC-3′ and 5′-CTGCATGAGCAGGACCTCCAG-3′), *Pgd* (5′-AGGACTCGTGGCGCGAGGTG-3′ and 5′-GGAATGTGTGAA CGGGAAAGTGGAG-3′), and *adh* (5′-AAACTGGCCCCCAT TACCG-3′ and 5′-CAAGTCCAGTTTCCAGATG-3′). cDNA was synthesized using random (hexanucleotide) or specific primers. Primers were added to total RNA (1 μg) and heated to 70° for 10 min. First-strand cDNA synthesis was performed with SuperScript II reverse transcriptase (200 units; GIBCO BRL, Gaithersburg, MD) for 1 hr at 42°. Controls without RT were processed in parallel. The enzyme was heat inactivated at 70° for 15 min. Reverse transcription of several independently isolated RNA samples was done. Samples (2 μl) from the reverse transcription reaction were amplified with Taq polymerase in the presence of dATP-αP33. The linear range of amplification was determined in preliminary experiments, and, depending on transcript abundance, 18–30 cycles were performed. Amplification was done using touchdown PCR with a final annealing temperature of ~40° (depending on the primer set). PCR products were separated in 5% denaturing acrylamide gel and visualized and quantified using PhosphoImager Storm-840 (Amersham, Piscataway, NJ).

Cloning and sequencing: The fragments obtained by PCR were cloned into pGEM-T vector (Promega, Madison, WI). Sequencing was performed using big dye-termination reagents and ABI/PE 377 automated sequencers. Sequences were analyzed by BLAST searches.

Chromatin immunoprecipitation: Chromatin immunoprecipitation (ChIP) was performed according to described procedure (Chanas *et al.* 2004). The analysis of DNA obtained in ChIP was done by semiquantative radioactive PCR as described above. Signals were quantified by PhosphorImager Storm-840. The ratio of signals from precipitated satellite DNA and DNA of the control *Pgd* gene *vs.* signals from input chromatin was calculated for *spn-E*^{1}*/spn-E*^{1} and +*/spn-E*^{1} ovaries.

RESULTS AND DISCUSSION

1.688 satellite DNA is transcribed in germinal tissues of *D. melanogaster* and is under the control of RNAi in ovaries: We have analyzed the transcriptional status of 1.688 satellite DNA in germinal tissues (ovaries and testes) of flies carrying mutations in the *spn-E* and *aubergine* genes. We compared 1.688 satellite transcript abundance in the homozygotes *spn-E*^{1}*/spn-E*^{1} and *aub*^{QC42}*/aub*^{QC42} and in the *trans*-heterozygote *spn-E*^{1}*/spn-E*$^{hls\text{-}03987}$ with corresponding heterozygous flies. The evaluation of transcript abundance was monitored by reverse transcription using random primers followed by semiquantitative PCR with primers specific for a given 1.688 satellite DNA subfamily. PCR amplification and gel analysis revealed a major target band as well as minor bands corresponding to nontarget satellite subfamilies or damaged satellite copies (Figure 1B), since we failed to design primers strictly specific to a definite subfamily of 1.688 satellites because of a high redundancy of their sequences. Therefore, we considered only the bands corresponding to the expected size of a target satellite subfamily in RT–PCR and ChIP (see below) analysis (Figure 1B).

Transcription of 1.688 satellite DNAs was detected both in ovaries and in testes (Figure 2A). 1.688 satellite transcript abundance normalized to a *rp49* transcript level shows its increase in *spn-E*^{1}*/spn-E*^{1}, *spn-E*^{1}*/spn-E*$^{hls\text{-}03987}$, and *aub*^{QC42}*/ aub*^{QC42} ovaries relative to ovaries from heterozygous flies (Figure 2A). The increase of satellite expression in ovaries is twofold for the *X*-linked 359-bp subfamily and achieves a fivefold increase for autosomal 260- and 361-bp subfamilies (Figure 2B). In ovaries, the transcript abundances of all subfamilies are maintained on a roughly equal and lower level. This result suggests a low level of the 359-bp subfamily transcripts in ovaries compared to autosomal pericentromeric subfamilies, taking into account that the *X*-linked array of 359-bp repeats is represented by several thousand copies and constitutes the major part of the 1.688 satellite DNA

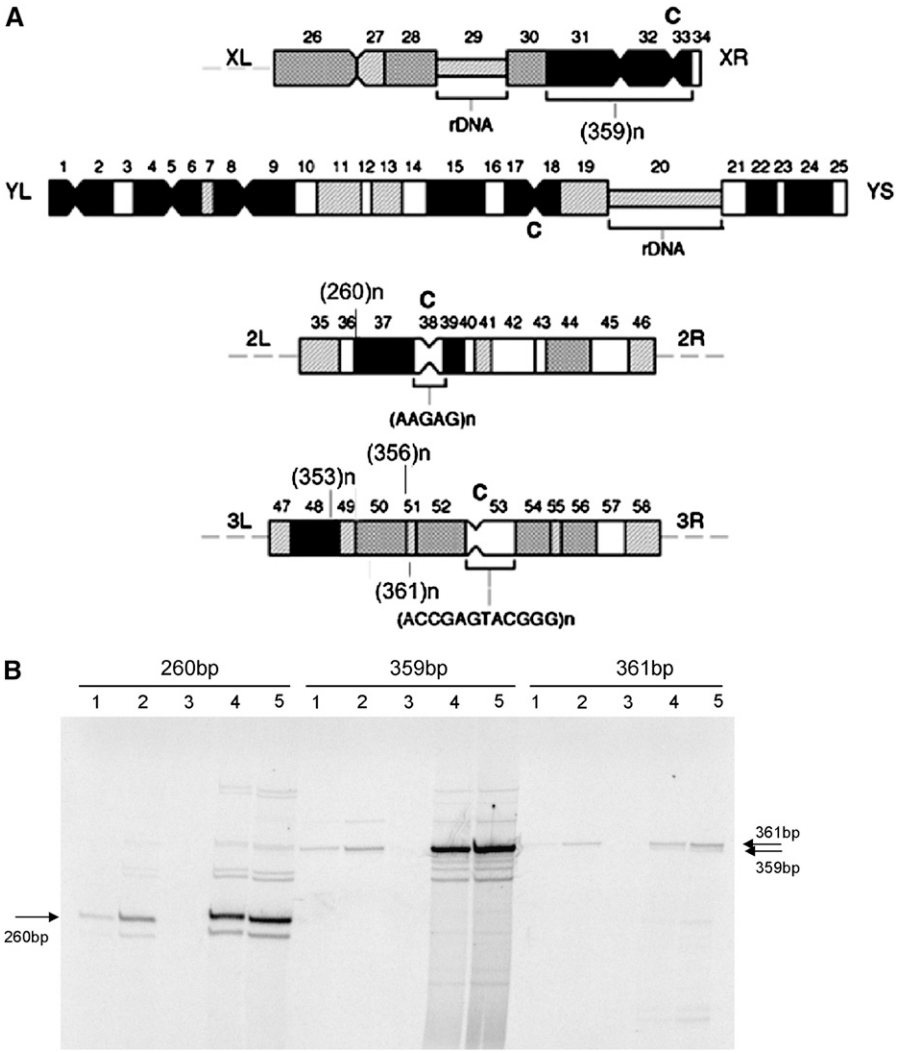

FIGURE 1.—(A) Schematic of 1.688 satellite locations in heterochromatin of large *D. melanogaster* chromosomes. Heterochromatic segments are indicated according to GATTI *et al.* (1994). "C" denotes centromeres; centromere-forming and ribosomal repeats are shown. 1.688 satellite subfamilies are denoted by characteristic size of repeat unit. (B) Gel analysis of PCR-amplified products obtained with primers for 260-, 361-, and 359-bp 1.688 satellite subfamilies. The designed primers are not strictly specific and amplify many bands corresponding to nontarget satellite subfamilies or damaged satellite copies; however, major target bands may be identified. In all experiments, we have analyzed only target bands. Lanes 1 and 2, ovaries; lane 3, no-RT, *spn-E*^{1}*/spn-E*^{1} ovarian RNA; lanes 4 and 5, testes; lanes 1 and 4, +*/spn-E*^{1}; lanes 2 and 5, *spn-E*^{1}*/spn-E*^{1}.

(HSIEH and BRUTLAG 1979). In testes, the level of 1.688 transcripts in *spn-E*^{1}*/spn-E*^{1} flies does not significantly change due to *spn-E*^{1} mutation (Figure 2A).

To extend the analysis of the effects of *spn-E*^{1} mutation on satellite transcription, the cDNAs obtained from *spn-E*^{1}*/spn-E*^{1} and +*/spn-E*^{1} ovaries were PCR amplified with primers for the 361-bp subfamily, cloned, and sequenced (Figure 2C). Owing to a high similarity of 1.688 satellite subfamilies, we also obtained clones related to the other subfamilies. We detected strong differences between the representatives of cDNA sets from *spn-E*^{1}*/spn-E*^{1} and +*/spn-E*^{1} ovaries (Figure 2C). cDNA clones from +*/spn-E*^{1} ovaries are represented mainly by 1.688 satellites located at the 9B1 euchromatic region of the *X* chromosome (~90%). The repertoire of the cDNA set from *spn-E*^{1}*/spn-E*^{1} ovaries is more diverse. The appearence of the target 361-bp cDNAs is the most pronounced effect of the *spn-E*^{1} mutation: no 361-bp clones from +*/spn-E*^{1} ovaries were detected, whereas approximately half of the clones from *spn-E*^{1}*/spn-E*^{1} ovaries corresponds to the 361-bp subfamily. Interestingly, three of four previously cloned 1.688 satellite siRNAs (ACUAAUUACCAGCGCUAACGAUCCCUAU, UUAGGGAAAUUAGUUUUGG, and UGAUGACCGA AAUUUGGAAAAACGG) (ARAVIN *et al.* 2003) originated from the 361-bp subfamily transcripts. The *spn-E*^{1} mutation leads to the increase of cDNA clones of pericentromeric autosomal satellites, including the 260- and 356-bp subfamilies. No effect of the *spn-E*^{1} mutation on the *X*-linked 359-bp subfamily was detected. This result may not be attributed to a low efficiency of primers used to amplify the 359-bp repeats, because the effective amplification of 359-bp satellites was attained in ChIP experiments (data not shown).

Thus, the extent of the effect of the *spn-E*^{1} mutation was more pronounced for pericentromeric autosomal subfamilies (260 and 361 bp) than for the *X*-linked 359-bp subfamily. These results indicate the differential transcriptional responses of euchromatic, centromeric, and pericentromeric subfamilies of 1.688 satellite DNA, owing to the disturbance of the RNAi mechanism.

Both strands of the 1.688 satellite DNA are transcribed: It is thought that the formation of double-stranded RNA is crucial for RNAi-dependent regulation

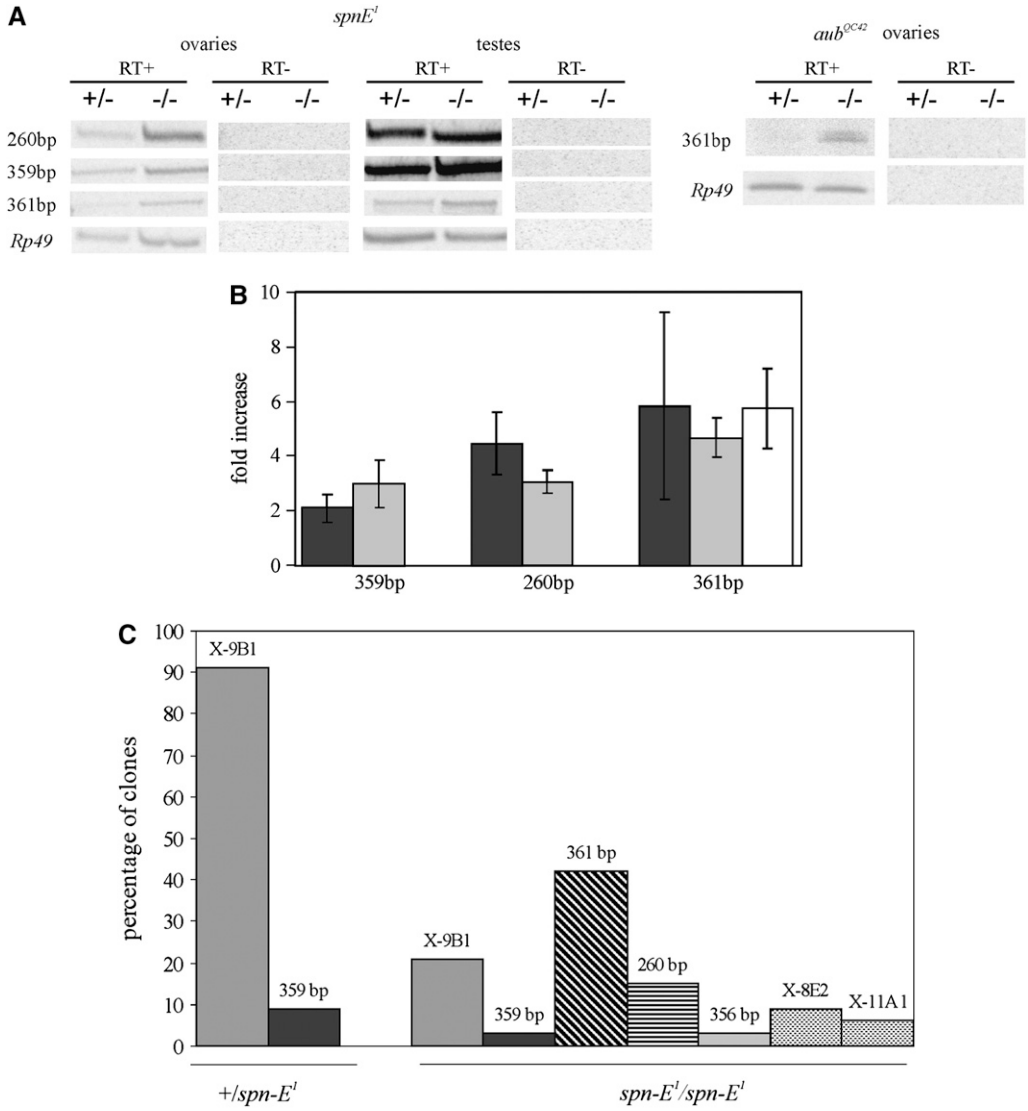

Figure 2.—Analysis of 1.688 satellite transcription in germinal tissues. (A) RT–PCR analysis of transcription of 1.688 satellite subfamilies in ovaries and testes of homozygous (−/−) and heterozygous (+/−) *spn-E*^{1} and *aub*^{QC42} flies. Transcription of the ubiquitously expressed *rp49* gene was used as a loading control. Amplification of RT controls (RT−) without reverse transcriptase gives no signals. (B) Mean ratios of satellite transcript abundance normalized to the *rp49* transcript level show the increase of 1.688 satellite transcript abundance in *spn-E*^{1}/*spn-E*^{1} (dark shading), *spn-E*^{1}/*spn-E*$^{hls\text{-}03987}$ (light shading), and *aub*^{QC42}/*aub*^{QC42} (no shading) ovaries relative to ovaries of heterozygous flies. (C) Cloning, sequencing, and annotating of PCR-amplified cDNAs from *spn-E*^{1}/*spn-E*^{1} and +/*spn-E*^{1} ovaries show transcriptional activation of various groups of 1.688 satellites in the presence of the *spn-E*^{1} mutation. PCR amplification of cDNAs was performed in exponential phase with primers for the 361-bp subfamily. However, several other subfamilies were also detected: *X*-linked euchromatic regions 9B1, 8E2, and 11A1; the *X*-linked 359-bp subfamily; and autosomal 260- and 356-bp subfamilies. The *y*-axis presents percentages of clones. The number of clones is 23 for +/*spn-E*^{1} and 33 for *spn-E*^{1}/*spn-E*^{1} flies. The sequences of all clones are in supplemental Table 1 (http://www.genetics.supplemental/).

of repeats. The emergence of double-stranded RNA in *S. pombe* is a primary step in siRNA formation, but the subsequent self-maintenance of the siRNA pool is most likely ensured by RNA-dependent RNA polymerase (RdRP) (Volpe *et al.* 2002). Persistent formation of transcripts from both DNA strands ensures the maintenance of a double-stranded RNA level in humans and flies lacking RdRP and may be considered a prominent feature of heterochromatic repeats in eukaryotes lacking RdRP. It has been recently shown that cellular dsRNAs in mammals are mainly produced by tandem repeat transcription, but not as a result of the transcription of repeated interspersed elements (Martens *et al.* 2005).

We detected transcripts corresponding to both strands of the 260-, 361-, and 359-bp subfamilies (Figure 3). The *spn-E*^{1} mutation causes the increase of transcript abundance, corresponding to both strands of satellites of the 260- and 359-bp subfamilies and to only a single strand of the 361-bp subfamily (Figure 3). The presence of transcripts from both strands of satellite DNA may lead to dsRNA formation.

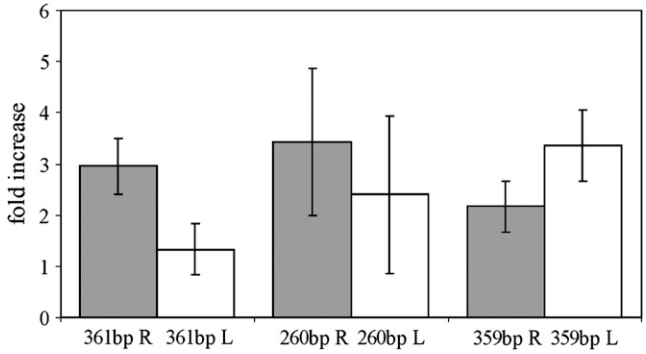

Figure 3.—RT–PCR analysis of strand-specific 1.688 satellite transcription in *spn-E*^{1}/*spn-E*^{1} and +/*spn-E*^{1} ovaries revealed transcription of both strands. The diagram presents the ratios of satellite transcript abundance in homozygous *spn-E*^{1}/*spn-E*^{1} ovaries to *spn-E*^{1}/+ heterozygotes. Strand-specific primers (R, "right," and L, "left") for each subfamily are the same as in randomly primed RT–PCR analysis. *Adh* gene product was used as a loading control.

***spn-E*^{1} mutation leads to the increase of the H3-AcK9 mark and TAF1 amount in the chromatin of autosomal pericentromeric 1.688 satellites:** Histone lysine modifications, including lysine 9 of histone H3, mark functionally distinct chromatin regions. Dimethylation of Lys9 (H3-diMeK9) followed by binding of heterochromatic protein 1 (HP1) or its homologs is generally thought to be a heterochromatic feature (Lachner *et al.* 2001; Nakayama *et al.* 2001). Acetylation at Lys9 (H3-AcK9) is a mark of active euchromatic regions (Turner 2000; Jenuwein and Allis 2001). Volpe *et al.* (2002) have reported a loss of H3-diMeK9 and Swi6, the HP1 homolog, at the centromeric repeats in *S. pombe* as a result of mutations in the RNAi system. It has been shown that in *D. melanogaster* mutations in the *spn-E* gene and in the other genes of the RNAi system, *piwi* and *aubergine* produce a drastic effect on heterochromatin state in somatic tissues, resulting in a considerable loss of H3-diMeK9 and redistribution of heterochromatic proteins HP1 and HP2 away from the chromocenter (Pal-Bhadra *et al.* 2004).

To test if *spn-E*^{1} mutation causes changes in the chromatin of 1.688 satellite DNA, we performed ChIP analysis of ovarian chromatin in *spn-E*^{1}/*spn-E*^{1} and +/*spn-E*^{1} flies with antibodies against H3-diMeK9, H3-AcK9, H3-triMeK9, H4-tetraAc (H4, acetylated at lysines 5, 8, 12, 16), and TAF1, a component of the RNA polymerase II transcriptional complex (Wassarman *et al.*

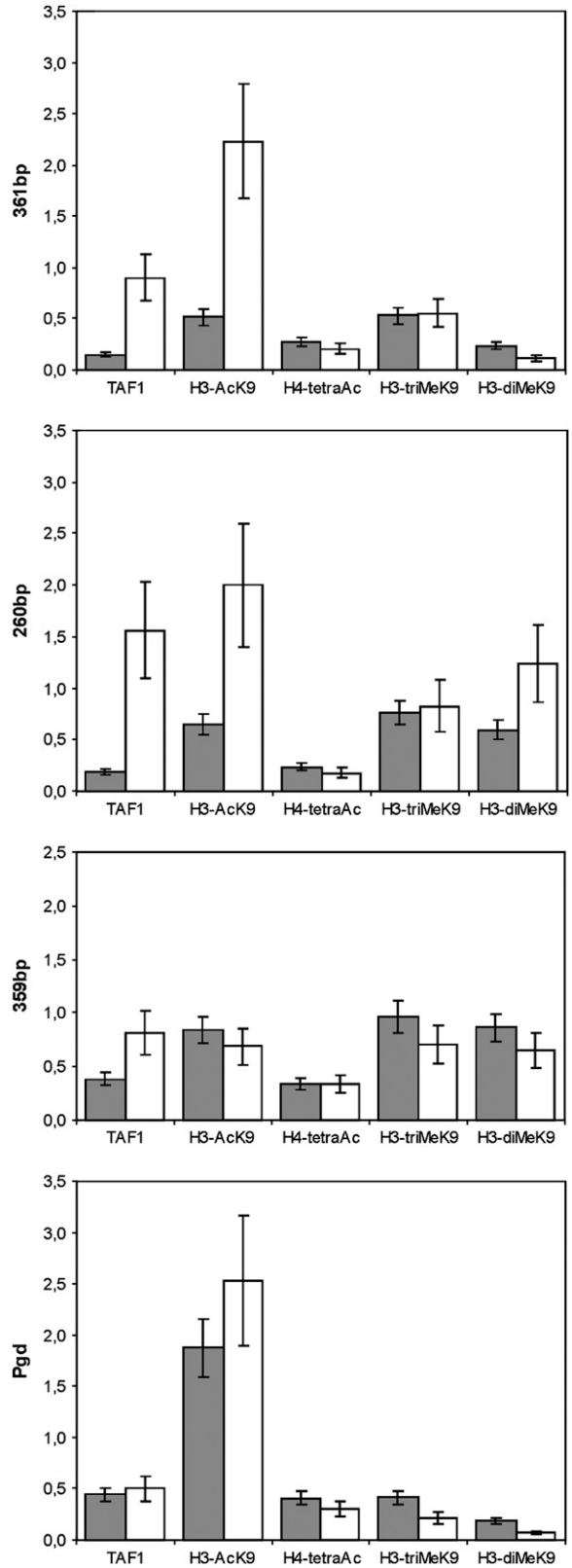

Figure 4.—ChIP analysis of 1.688 satellite chromatin in +/*spn-E*^{1} and *spn-E*^{1}/*spn-E*^{1} ovaries, using antibodies against H3-diMeK9, H3-AcK9, H3-triMeK9, H4-tetraAc, and TAF1. DNA purified from input ovarian chromatin or precipitated with antibodies was analyzed by semiquantitative PCR. To normalize target DNA signal, we used DNA of the transcribed region of the ubiquitously expressed housekeeping *Pgd* gene. Shaded columns refer to +/*spn-E*^{1} and open columns to *spn-E*^{1}/*spn-E*^{1} ovaries. DNA precipitated with antibodies was PCR amplified in exponential phase with primers for the corresponding subfamily, separated on polyacrylamide gel, and quantified by PhosphorImager. Values are indicated as the percentage of precipitation relative to the input. The experiment was performed two times.

2000) (Figure 4). ChIP data show the increase of accumulation of the H3-AcK9 mark and TAF1 on chromatin of tested autosomal pericentromeric repeats, owing to *spn-E*^{1} mutation (Figure 4). The four- to fivefold increase of TAF1 occupancy of the 361- and 260-bp subfamilies of satellites was revealed in homozygous *spn-E*^{1} flies as compared with heterozygous ones, while difference in TAF1 amount on 359-bp repeats was shown to be insignificant (Figure 4). We detected no changes in H3-MeK9 marks of 1.688 satellite chromatin. No significant effects for all tested antibodies were detected for the chromatin of the *X*-linked 359-bp subfamily. These data correlate with the level of transcript abundance increase, owing to *spn-E*^{1} mutation, that is stronger for pericentromeric satellites (361 and 260 bp) than for the 359-bp subfamily (Figure 2).

Together, these results indicate that 1.688 satellite transcription is most probably executed by RNA polymerase II and is modulated by a RNAi-dependent mechanism of silencing on a chromatin level.

Conclusion: Transcription of the 1.688 satellite DNA in the *D. melanogaster* genome has not been previously shown. We detected transcription of 1.688 satellite subfamilies in the germline and the increase of their transcript abundance in ovaries of flies carrying mutations in the *spn-E* and *aub* genes. These results reveal the participation of the RNAi system in silencing of the 1.688 satellite DNA. The effect of the *spn-E*^{1} mutation is more pronounced for satellite arrays localized in pericentromeric regions of autosomes (361 and 260 bp) than for the *X*-linked centromeric cluster (359 bp). These observations are in accordance with the results of ChIP analysis showing the increase of TAF1 occupancy of the 361-bp subfamily but not of the 359-bp subfamily. Our results suggest that differences in the RNAi-dependent regulation of 1.688 satellite transcription may be correlated with a specificity of their chromatin organization. This difference between centromeric and pericentromeric satellites may be related to their function in kinetochore formation and chromosome cohesion, respectively. The significance of the correlation between functional peculiarities of satellite subfamilies and the dependence of their chromatin properties on RNAi needs to be unveiled in further studies.

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