Maternal impairment of transposon regulation in *Drosophila melanogaster* by mutations in the genes *aubergine*, *piwi* and *Suppressor of variegation 205*

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(Received 24 June 2010 and in revised form 14 August 2010)

Summary

TP5, a *P* element inserted in the telomere-associated sequences of the X chromosome, represses the excision of other *P* elements in the germ line through a combination of maternal and zygotic effects. The maternal component of this repression is impaired by heterozygous mutations in the *aubergine* and *Suppressor of variegation 205* genes; one mutation in the *piwi* gene also appears to impair repression. In the female germ line, the level of TP5 mRNA is increased by these impairing mutations. The impairing *aubergine* and *piwi* mutations also increase the level of germ-line mRNA from *CP*, a transgene that encodes the *P*-element transposase; however, the *Suppressor of variegation 205* mutation does not. These findings are discussed in terms of a model of *P*-element regulation that involves post-transcriptional and chromatin re-organizing events mediated by maternally transmitted small RNAs derived from the telomeric *P* element.

1. Introduction

Transposable elements are present in the genomes of many organisms, including model genetic species such as *Drosophila melanogaster*. The population of transposons in this organism comprises elements that move through the agency of a transposase enzyme (the cut-and-paste transposons) and elements that move by producing DNA copies of their RNA transcripts (the retrotransposons). Among the hundred or so different types of transposons in the *Drosophila* genome, only a few have been intensively studied. One of them, the cut-and-paste transposon called the *P* element, has become a valuable tool for genetic analysis in *Drosophila*. It has also provided important insights into how *Drosophila* transposons are regulated.

*P* elements were discovered through their involvement in the phenomenon of hybrid dysgenesis, a syndrome of abnormal germ-line traits that occurs non-reciprocally in the offspring of crosses between certain types of *Drosophila* strains (Kidwell *et al.*, 1977; Engels, 1989). *P* strains contain *P* elements in their genomes, whereas *M* strains do not. Crosses between *P* males and *M* females produce dysgenic offspring, whereas crosses between *M* males and *P* females and between *P* males and *P* females generally do not. The traits of hybrid dysgenesis – agametic sterility, increased frequencies of mutation and chromosome breakage, transmission ratio distortion and chromosome non-disjunction – occur in the hybrid offspring of *P* males and *M* females because the *P* elements that are contributed by the males in these crosses are not repressed in the offspring. In the other crosses, a maternally contributed state called the *P* cytotype represses *P*-element activity in the offspring (Engels, 1979).

Recent advances in genomic analysis have implicated small RNAs in the repression of transposon activity (Brennecke *et al.*, 2007, 2008; Li *et al.*, 2009). These RNAs associate with the Piwi class of proteins, which in *Drosophila* includes the denominative Piwi and two other members, Aubergine and Argonaute3. These RNAs are therefore called Piwi-interacting or piRNAs. Certain loci in the *Drosophila* genome produce piRNAs. One especially productive locus is situated in the telomere-associated sequences (TAS)
at the end of the left arm of the X chromosome (XL). When P elements are inserted in this locus, they participate in the generation of piRNAs. Because the P-specific piRNAs derived from this and other loci are transmitted maternally through the egg cytoplasm (Brennecke et al., 2008), they are thought to be the physical basis of the P cytotype (Jensen et al., 2008). In the offspring of crosses involving P females, antisense piRNAs may repress hybrid dysgenesis by targeting P-element mRNAs, including those that encode the P transposase, for destruction. P-specific piRNAs may also guide protein complexes to P elements inserted at diverse locations in the genome to prevent their movement.

The genetic analysis of telomeric P elements has revealed the important role they play in P-element regulation (Ronsseray et al., 1991, 1993, 1996, 1998; Marin et al., 2000; Stuart et al., 2002; Simmons et al., 2004; Niemi et al., 2004; Thorp et al., 2009; Belinco et al., 2009). We have endeavoured to extend this analysis by examining the effects of mutations in the genes aubergine (aub), piwi and Suppressor of variegation 203 [Su(var)203] on the maternal component of this regulation. The genes aub and piwi encode Piwi-type proteins that associate with piRNAs (Brennecke et al., 2007); Su(var)203 encodes a protein called heterochromatin protein 1 (HP1), which is involved in chromatin organization (James et al., 1989). Heterozygous mutations in all three genes have been shown to impair the repression of hybrid dysgenesis (Ronsseray et al., 1996; Reiss et al., 2004; Haley et al., 2005; Simmons et al., 2007; Belinco et al., 2009).

In this paper, we address how the aub, piwi and Su(var)205 mutations impair P-element regulation. First, by monitoring excisions of particular P elements in the germ line, we document that this regulation depends on a maternal component. Second, using the excision assay, we investigate the maternal effects of aub, piwi and Su(var)205 mutations on P regulation. Finally, to elucidate the underlying molecular mechanism, we examine the effects of these mutations on the expression levels of specific P-element mRNAs in the female germ line.

2. Materials and methods

(i) Drosophila stocks and husbandry

Information on the special chromosomes and mutant alleles used in the experiments is available on the Flybase website, in Lindsley & Zimm (1992), or in other references cited in the text. Experimental cultures were reared in vials on a standard cornmeal–molasses–dried yeast medium; the culturing temperatures are specified in the text.

TP5 is a 1.8-kb-long P element inserted in the TAS at the left end of an X chromosome marked with the tightly linked recessive mutation w (white eyes); see Stuart et al. (2002) for the isolation and characterization of this telomeric P element. The TP5 w X chromosome was incorporated into a Gla/CyO stock, and later, into stocks carrying aub, piwi or Su(var)205 mutations balanced with the CyO chromosome II (Belinco et al., 2009). Control stocks were subsequently established by removing the mutations from the genotype (Belinco et al., 2009). A TP5 w snw; Gla/CyO stock was established by exchanging the CyO balancer for the Cy Roi balancer in a TP5 w snw; Gla/Cy Roi stock (Simmons et al., 2007) and a control w snw; Gla/CyO stock was established in the same way. In all these stocks, the CyO chromosome carried P(SB7), an insertion of a P transgene that contains the Drosophila mini-white gene sandwiched between the terminal inverted repeats of a vertebrate transposon called Sleeping Beauty (Ivics et al., 1997). This insertion, abbreviated SB7, confers a dark orange eye colour on flies in which the native white gene is mutant, and it becomes mobile when a source of the P transposase is present in the genome.

P(ry), Δ2-3)99B, a P transgene abbreviated Δ2-3, is a stable source of the P transposase inserted on chromosome III (Robertson et al., 1988). H(hsp/CP)2, a hobo transgene abbreviated CP, is another stable source of the P transposase inserted in chromosome II (Simmons et al., 2002a). This transgene produces the P transposase only in the germ line. Δ2-3, by contrast, produces it in both the soma and the germ line. The somatic activity of Δ2-3 can make it difficult to score phenotypes that reveal P-element activity. Consequently, when Δ2-3 was used to induce P activity, these phenotypes were scored in genotypes that repress somatic transposase activity (Robertson & Engels, 1989).

(ii) P-element excision assay

The singed-weak (snw) allele is a double P-element insertion mutation of the X-linked singed bristle (sn) gene (Roila et al., 1988). This allele causes a mild malformation of the bristles on the adult cuticle. In the presence of the P transposase, either of the two P elements inserted in snw can be excised. Excision of one element produces a more extreme mutant phenotype (snw), whereas excision of the other element produces a pseudo-wild phenotype (snw+). Germ-line P-excisions from snw were detected by crossing an snw male to females with attached X chromosomes. The male’s sons, which inherited their X chromosome patroclinously, were then scored for the three possible bristle phenotypes. The proportion of snw+ and snw flies among these sons (a statistic referred to as the ‘snw mutability’) was used to quantify the male’s germ-line P-excision activity.
Summary statistics were calculated after pooling the data from the two cultures. These cultures were scored for their bristle and eye colour phenotypes on days 14 and 17 after the cultures were established.

The (TP5) w sn w; CyO, SB7/+; ∆2-3/+ males for these tests were obtained from “reciprocal” crosses between (TP5) w sn w; CyO, SB7/Gla and w; ∆2-3 strains at 18 °C: (TP5) w sn w; CyO, SB7/Gla females × w; ∆2-3 males (maternal transmission) and (TP5) w sn w; CyO, SB7/Gla males × C(1)DX, y w f; ∆2-3 females (paternal transmission). Each tested male was crossed to four C(1)DX, y f females from a P strain at 25 °C. After 5 days the flies were transferred to a fresh culture. The sons of both cultures were scored for their bristle and eye colour phenotypes on days 14 and 17 after the cultures were established. Summary statistics were calculated after pooling the data from the two cultures.

Table 1. Germ-line P-element and P-transgene excision in males carrying a maternally or a paternally inherited telomeric TP5 element

| X chromosome | Transmission | No. of tests | No. of sons | sn w mutability | No. of curly sons | SB7 excision frequency a b |
|--------------|--------------|--------------|-------------|-----------------|-------------------|--------------------------|
| w sn w       | Maternal     | 45           | 2561        | 0.524 ± 0.019   | 1171              | 0.324 ± 0.022          |
| w sn w       | Paternal     | 39           | 1837        | 0.507 ± 0.027   | 824               | 0.298 ± 0.024          |
| TP5 w sn w   | Maternal     | 44           | 2084        | 0.113 ± 0.014   | 932               | 0.063 ± 0.015          |
| TP5 w sn w   | Paternal     | 47           | 2702        | 0.555 ± 0.019   | 1270              | 0.306 ± 0.018          |

The (TP5) w sn w; CyO, SB7/+; ∆2-3/+ males for these tests were obtained from “reciprocal” crosses between (TP5) w sn w; CyO, SB7/Gla and w; ∆2-3 strains at 18 °C: (TP5) w sn w; CyO, SB7/Gla females × w; ∆2-3 males (maternal transmission) and (TP5) w sn w; CyO, SB7/Gla males × C(1)DX, y w f; ∆2-3 females (paternal transmission). Each tested male was crossed to four C(1)DX, y f females from a P strain at 25 °C. After 5 days the flies were transferred to a fresh culture. The sons of both cultures were scored for their bristle and eye colour phenotypes on days 14 and 17 after the cultures were established. Summary statistics were calculated after pooling the data from the two cultures.

a Unweighted average frequency of phenotypically sn w and sn w sons among all sons scored ± se.
b Unweighted average frequency of white-eyed sons among curly-winged sons scored ± se.

(iii) P-transgene excision assay

Flies with the CyO, SB7 chromosome and a mutant native w gene have curly wings and coloured eyes. If the SB7 transgene is excised from the CyO chromosome in a male’s germ line, some of the male’s curly-winged offspring will have white eyes. The proportion of white-eyed curly flies among all the curly offspring can therefore be used as a measure of P-transgene excision activity in the male’s germ line. This statistic will, of course, underestimate the true excision activity because some of the excised SB7 transgenes may re-integrate, either on the CyO chromosome or on another chromosome, and then be passed on to the offspring, which will develop coloured eyes. No attempt was made to analyse such reintegration events in the experiments reported here.

(iv) Statistical analyses

Excision frequencies for the sn w P elements and the SB7 transgene were calculated independently for each replicate culture in an experimental group and the unweighted average frequency among the replicates was used to characterize that group. The standard error (se) for this average was computed empirically from the variance among replicates. Statistical differences between groups were assessed by performing t or z tests.

(v) RNA isolation and reverse transcription (RT)–PCR

RNA was isolated from groups of 20 virgin females using TRIZOL (Invitrogen) according to the supplier’s instructions. The RNA was reverse transcribed into cDNA using the ThermoScript reverse transcriptase (Invitrogen) and an oligo-dT primer, and the resulting cDNA was amplified by the PCR over 30 cycles using appropriate oligonucleotide primers and temperature profiles. The detailed methods for RT–PCR and the P-element primer sequences are given in Jensen et al. (2008). The sequences of the primers that were used to amplify singed cDNA are: sn-u 5'-CGTATCTCTTGGGTCTATCAACG-3' and sn-d 5'-CTGGTCATCTGTTTGCCACCTC-3'. These primers anneal to segments of different exons in the singed gene. The PCR profile for amplification of singed cDNA was 3 min at 92 °C, 2 min at 60 °C and 3 min at 72 °C followed by 29 cycles consisting of 1 min at 92 °C, 2 min at 60 °C and 3 min at 72 °C. All PCR products were analysed in 1% agarose gels by electrophoresis at 70 volts.

3. Results

(i) A maternal component in the repression of P-element and P-transgene excisions in males

The capacity for P-element regulation in the male germ line has previously been studied by monitoring the frequency of transposase-induced P-element excisions from sn w, a double P-insertion allele of the X-linked singed locus. These studies have indicated that regulation occurs when the males inherit an X-linked telomeric P-element maternally; however, when they inherit the telomeric P-element paternally, regulation is lost (Simmons et al., 2004). As a basis for further genetic and molecular analysis of P-element regulation, we confirmed the requirement for maternal inheritance of the telomeric P-element by simultaneously monitoring excisions of the P elements from sn w and excisions of the P transgene, SB7, from the CyO balancer chromosome II. These excisions were induced in males by the ∆2-3 transposase source inserted in chromosome III, and regulatory capacity was provided by the telomeric P element TP5. The results of this two-locus assay for repression of P excisions are summarized in Table 1.
Mutant stocks with and without the TP5 element were used in this experiment. The tested (TP5) w: CyO, SB7/+; Δ2-3/+ males were obtained by crossing (TP5) w: CyO, SB7/mutation females with w; Δ2-3 males at 18 °C. Each tested male was crossed to three females from the Harwich w P strain at 25 °C and the curly-winged offspring were scored for their eye colour phenotype on day 14. Although all the tested males were mosaic for eye colour, their offspring, which inherited the P genetic background from their mothers, were not. Asterisks indicate excision frequencies that are significantly greater than the frequency for the Gla mutation at the 5% level.

\[ a \] Unweighted average frequency of white-eyed flies among curly-winged flies ± se.

In control tests, in which TP5 was absent, the frequency of P-element excisions, measured by the mutability of sn\(^w\), was around 0·5, and the frequency of P-transgene excisions, measured by the loss of the SB7 transgene from the CyO chromosome, was around 0·3, regardless of the parental origin of the transposase targets (sn\(^w\) and SB7) or the transposase source (Δ2-3). In males that had inherited TP5 maternally, these excision statistics were dramatically reduced (sn\(^w\) mutability = 0·11 and SB7 excision frequency = 0·06), but in males that had inherited TP5 paternally, they were essentially the same as those from males that did not carry TP5. The difference between the two types of TP5-bearing males cannot be attributed to cytoplasmic transmission of P transposase activity in the cross where Δ2-3 was inherited maternally and TP5 was inherited paternally because no such transmission is seen with the Δ2-3 transposase source (Simmons et al., 2002). Repression of both P-element and P-transgene excisions in the male germ line therefore requires the maternal inheritance of the TP5 element. Other studies employing the sn\(^w\) mutability assay have shown that repression of P excisions cannot be explained by a simple maternal effect of TP5 (Stuart et al., 2002; Thorp et al., 2009); rather, it requires the combined maternal and zygotic effects of this telomeric P element.

If it should also be noted that all the males tested in this assay exhibited pronounced mosaicism for the bristle and eye colour phenotypes, even when they inherited TP5 maternally. This observation confirms published evidence that TP5 does not repress P excisions induced by the transposase that Δ2-3 produces in somatic tissues (Stuart et al., 2002; Simmons et al., 2004). In the sons of these males, however, the somatic mosaicism was suppressed by the P genetic background inherited from their mothers.

(ii) Maternal effects of mutations on repression of P excisions in males

We used the P-transgene excision assay to determine if the maternal component of P-element regulation requires the proteins encoded by the genes aub, piwi and Su(var)205, which are all located in chromosome II. We monitored excisions of the SB7 transgene in the sons of TP5 w females that were heterozygous for the CyO, SB7 chromosome II and a mutation in one of these genes. These TP5 w: CyO, SB7/mutation females were crossed to w males homozygous for a source of the P transposase to obtain the TP5 w: CyO, SB7/+ males that were tested. SB7 excisions in the germ lines of these males should be repressed by the zygotic and maternal effects of the maternally inherited TP5 element. However, if the heterozygous mutation present in the mother of each tested male depletes a protein that is essential for the maternal effect, the repression mechanism would be impaired and the SB7 excision frequency would increase.

We tested two aub mutations, two piwi mutations and one Su(var)205 mutation in two different experiments, one using the Δ2-3 transposase source (Table 2) and the other using the CP transposase source (Table 3). As a control, we used a stock carrying the Gla mutation, which has not been implicated in any aspect of transposon regulation. In both experiments, we also tested stocks that did not carry the TP5 element to determine if the various mutations influenced transgene excision frequency in the absence of P-element regulation.

In the tests comprising males that did not carry TP5 (left sides of Tables 2 and 3), none of the mutations affected the frequency of SB7 excisions in the germ line. With the Δ2-3 transposase source, these frequencies ranged from 0·31 to 0·42 and the control

Table 2. Maternal effects of heterozygous mutations on TP5-mediated repression of Δ2-3-induced P-transgene excision in the male germ line

| Mutation            | No. of males | No. of flies | Excision frequency\(^a\) | No. of males | No. of flies | Excision frequency\(^a\) |
|---------------------|--------------|--------------|--------------------------|--------------|--------------|--------------------------|
| Gla                 | 31           | 1054         | 0·342 ± 0·022            | 32           | 1555         | 0·088 ± 0·013            |
| aub\(^{G,c,a}\)     | 31           | 1263         | 0·310 ± 0·024            | 32           | 1415         | 0·287 ± 0·020*           |
| aub\(^{G,p,au}\)    | 32           | 1270         | 0·408 ± 0·018            | 30           | 1324         | 0·343 ± 0·026*           |
| piwi\(^{G}\)        | 31           | 1080         | 0·377 ± 0·021            | 31           | 9447         | 0·038 ± 0·011            |
| piwi\(^{G}\)        | 29           | 1267         | 0·416 ± 0·027            | 19           | 456          | 0·057 ± 0·014            |
| Su(var)205\(^{G}\)  | 30           | 800          | 0·416 ± 0·029            | 27           | 651          | 0·246 ± 0·037*           |

\(a\) Unweighted average frequency of white-eyed flies among curly-winged flies ± se.
frequency was 0.34, and with the CP transposase source, they ranged from 0.27 to 0.36 and the control frequency was 0.33. In the presence of a maternally inherited TP5 element, the control SB7 excision frequencies were significantly reduced – to 0.09 in the experiment with the Δ2-3 transposase source and to 0.03 in the experiment with the CP transposase source. Thus, as expected, the maternally inherited TP5 element repressed P-transgene excision in the male germ line. However, this repression was significantly impaired in the flies from the aubBCQ, aubC.P-Δ3 and Su(var)205 mutant stocks in both experiments and in the flies from the piwi mutant stock in the experiment with the CP transposase source. By contrast, the flies from the piwi mutant stock did not impair repression in either experiment.

It is important to note that the mutations tested in these experiments were not present in the males in which the P-transgene excisions occurred; rather, they were present in heterozygous condition in the mothers of these males. Thus, the impairment of repression seen in Tables 2 and 3 was apparently due to dominant maternal effects of the mutations.

To verify that the impairment was caused by the mutations and not by some other factor, we measured CP-induced SB7 excision frequencies in males derived from TP5 w; CyO, SB7/+ stocks from which the various mutations had been removed many generations previously. These mutation-free stocks are expected to have different genetic backgrounds than the stocks that carried the mutations because outcrossing was required to produce them. However, they have the same X chromosome and therefore serve as controls against the possibility that the regulatory function of the TP5 element was altered by random changes in the XL telomere, which is a genetically dynamic structure. The results, shown on the right in Table 3, demonstrate that the removal of the aubBCQ, aubC.P-Δ3 and Su(var)205 mutations from the original stocks restored repression of SB7 excision to a strong level. The impairment of repression that was seen when these mutations were present must therefore be due to the mutations themselves, not to some other factor. By contrast, removal of the piwi mutation did not strengthen repression significantly, and removal of the piwi mutation actually weakened it. Thus, other factors such as changes in the structure of XL may have influenced the regulatory function of the TP5 element in the piwi mutant stocks or in their mutation-free derivatives.

### (iii) Effects of mutations on levels of P-element mRNAs in the female germ line

As the aub and Su(var)205 mutations impair P-element regulation through dominant maternal
effects, we used RT–PCR to determine if, in heterozygous condition, these mutations affect the levels of P mRNAs in the maternal germ line; we also tested the piwi mutations for such effects. TP5 w; CyO, SB7/mutation females were crossed to w; CP males to produce the TP5 w/w; CP/mutation females that were used in this analysis. Control females were obtained by crossing w females with w; CP males. RNA was extracted from four samples of each of the various types of females, reverse transcribed into cDNA using an oligo-dT primer, and then the cDNA was amplified by PCR with other primers specific for mRNA from the singed gene (to assess RNA input levels) or mRNAs from the TP5 or CP elements. The results from these experiments, each utilizing the same four sets of cDNA samples, are presented in Figs 1 through 4.

Figure 1 shows the results of amplifying cDNAs derived from RT of mRNAs from the singed gene, which is expressed in the female germ line. The band intensities among the samples within each set are quite similar, indicating that roughly equal amounts of mRNA from the different genotypes were analysed.

Figure 2 shows the results of amplifying cDNAs derived from RT of CP mRNAs that retain the last P intron. Because this intron is not spliced out of P-element transcripts in the somatic cells, we refer to this class of mRNAs as ‘somatic.’ However, it is likely that these mRNAs are also produced in the germ line through incomplete splicing of CP transcripts; see Simmons et al. (2002a) for genetic evidence on this point. Figure 2 does not show any consistent differences in the intensities of the PCR products from the various samples that were analysed. The presence of the TP5 element or the presence of an aub, piwi, or Su(var)205 mutation therefore does not seem to affect the level of somatic CP mRNA in females. This finding is consistent with genetic evidence that TP5-mediated system for P regulation does not operate in somatic cells (Stuart et al., 2002; Simmons et al., 2004).

Figure 3 shows the results of amplifying cDNAs derived from RT of CP mRNAs that have lost the last P intron; these mRNAs are produced exclusively in the germ line. The specificity of this PCR for germ-line CP mRNA is due to the choice of primers, one
Table 4. Correlated genetic evidence for maternal effects of heterozygous repression of CP-induced P transgene excision in the male germ line

| Mutation          | No. of males | No. of flies | Excision frequencya |
|-------------------|--------------|--------------|---------------------|
| Gla               | 32           | 1162         | 0.011 ± 0.004      |
| aub\(^{QC42}\)    | 32           | 1010         | 0.110 ± 0.016*     |
| aub\(^{\Delta F a}\) | 30           | 1072         | 0.112 ± 0.023*     |
| piwi\(^{\varphi}\) | 31           | 873          | 0.104 ± 0.022*     |
| piwi\(^{\varphi}\) | 31           | 1105         | 0                  |
| Su(var)\(^{205}\) | 31           | 812          | 0.186 ± 0.020*     |
| Control (no TP5)  | 31           | 783          | 0.274 ± 0.020*     |

The (TP5)\(^{w}\); CyO, SB7/CP males for these tests were obtained from crosses between TP5\(^{w}\); CyO, SB7/mutation or w; CyO, SB7\(^{+/}\) (control) females and w; CP males at 25 \(^{\circ}\)C. Each male was crossed to three Harwich w females at 25 \(^{\circ}\)C and their curly-winged offspring were scored for the eye colour phenotype on day 14. The TP5\(^{w}\); mutation/CP or w; \(^{+/}\)CP (control) sisters of these tested males provided RNA for RT-PCR analysis. Asterisks indicate excision frequencies that are significantly greater than the frequency for the Glama mutation at the 5% level.

a Unweighted average frequency of white-eyed flies among curly-winged flies ± SEM.

spanning the first intron, which is in a region deleted in the TP5 element, and one spanning the last intron, which is removed only in germ-line cells. Three observations are noteworthy. First, in three of the four sets of gel images, the PCR product from the TP5\(^{w}\)/w; CP/Gla females (lane G) is clearly fainter than the product from the w/w; CP/+ control females (lane C). This observation confirms previous evidence that the presence of the regulatory TP5 element is associated with a reduction in the level of CP mRNA in the female germ line (Jensen et al., 2008; Thorp et al., 2009). Second, in all four sets of gel images, the PCR products from the TP5\(^{w}\)/w; CP/mutation males carrying the aub\(^{QC42}\), aub\(^{\Delta F a}\), piwi\(^{\varphi}\) or piwi\(^{\varphi}\) alleles are brighter than the products from the TP5\(^{w}\)/w; CP/Gla females (compare lanes Q, \(\Delta\) and P1 with lane G). The TP5-mediated loss of CP mRNA is therefore ameliorated in females with these mutant genotypes. Third, the PCR products from the TP5\(^{w}\)/w; CP/mutation females carrying the piwi\(^{\varphi}\) or Su(var)\(^{205}\) alleles (lanes P2 and S) are fainter than the products from the TP5\(^{w}\)/w; CP/Gla females in two of the four sets of gel images, and they are fainter than the products from the TP5\(^{w}\)/w; CP/mutation females carrying the aub\(^{QC42}\), aub\(^{\Delta F a}\) or piwi\(^{\varphi}\) alleles in all four sets of gel images. Thus, little or no amelioration of the loss of CP mRNA occurred in TP5\(^{w}\)/w; CP/piwi\(^{\varphi}\) or TP5\(^{w}\)/w; CP/Su(var)\(^{205}\) females.

Figure 4 shows the results of amplifying cDNAs derived from RT of TP5 mRNAs in the female germ line. Here, the specificity of the amplification is due to a primer that spans the deletion in the TP5 element; the other primer ensures that only germ-line cDNAs are amplified. TP5 mRNA cannot be produced by the control females (lane C), which lacked a TP5 element. Among the females that carried this element, those heterozygous for the Gla mutation (lane G) yielded little or no PCR product, indicating that they accumulate little TP5 mRNA – an observation that confirms previous evidence that P-element regulation is associated with a paucity of TP5 mRNA (Jensen et al., 2008; Thorp et al., 2009). The TP5\(^{w}\)/w females that were heterozygous for the piwi\(^{\varphi}\) mutation also accumulated little of this mRNA. By contrast, the TP5 females that were heterozygous for the aub\(^{QC42}\), aub\(^{\Delta F a}\), piwi\(^{\varphi}\) or Su(var)\(^{205}\) mutations yielded more of the TP5-specific PCR product in at least three of the four sets of gel images. Thus, in these females, the paucity of TP5 mRNA associated with P-element regulation is ameliorated.

To connect this analysis of P-element RNA with genetic data on regulation by the TP5 element inherited maternally from the various mutant stocks, we measured the frequency of SB7 excisions in the germ lines of the TP5\(^{w}\); CyO, SB7/CP brothers of the TP5\(^{w}\)/w; CP/mutation females from which the analysed RNA was extracted. The results, summarized in Table 4, demonstrate that the repression of CP-induced SB7 excision was impaired in males from the aub\(^{QC42}\), aub\(^{\Delta F a}\), piwi\(^{\varphi}\) and Su(var)\(^{205}\) mutations, but not in males from the piwi\(^{\varphi}\) stock, confirming previous findings (Table 3). Thus, the males with impaired P regulation corresponded to the females with elevated levels of germ-line TP5 mRNA. Impaired regulation is not, however, always associated with more expression of CP mRNA. Females heterozygous for three of the mutations – aub\(^{QC42}\), aub\(^{\Delta F a}\) and piwi\(^{\varphi}\) – did have elevated levels of CP mRNA in their germ lines, but females heterozygous for Su(var)\(^{205}\), which caused the most severe impairment of P regulation in males, did not.

4. Discussion

TP5-mediated repression of P-element excision in the male germ line has maternal and zygotic components, and both are essential to bring about the repressive state. Males that have inherited a TP5 element paternally do not repress P excision because they lack the maternal component, and males that have inherited cytoplasm but not TP5 maternally from heterozygous TP5/+ mothers do not repress because they lack the zygotic component (Stuart et al., 2002; Simmons et al., 2004; Thorp et al., 2009). These facts imply that some aspect of the TP5 function in the maternal germ line is necessary for the zygotic function of TP5 in the germ line of the male offspring.
Do aub, piwi, or Su(var)205 mutations affect the maternal component of P-element regulation in males? Our transgene excision experiments show that this regulation is impaired when the mothers of the males were heterozygous for a mutation in the aub gene. The Aub protein is normally present in the perinuclear cytoplasm of female germ cells, specifically in a region called the nuage (Brennecke et al., 2007). This protein appears to play an important role in producing, processing, or transporting piRNAs, including those generated from the locus in the TAS of the XL telomere into which TP5 is inserted (Brennecke et al., 2007, 2008). Females that are heterozygous for a mutant aub allele would be expected to have half as much Aub protein as homozygous wild-type females. Our genetic data show that this mutational depletion of Aub protein in the maternal germ line compromises the regulation of P elements in the germ lines of their male offspring. This compromised regulation cannot be due to a zygotic effect, the germ lines of their male offspring. This complementation of piRNAs. Transmission of P elements become active in the germ lines of these females, causing them to be sterile. Maternally transmitted I-element piRNAs appear to be responsible for repressing this sterility in females from the reciprocal cross (I female × R male) and in females from I strains; see also Brennecke et al. (2008). In this system, the Aub protein is evidently needed for the production, processing, or transport of these piRNAs, and for the elimination of I-element mRNAs.

The two piwi mutations analysed here had different – and inconsistent – maternal effects on P regulation in the male germ line. Our genetic data indicate that repression of P-transgene excision was impaired in males from the piwi\(^{d}\) stock but not in males from the piwi\(^{\delta}\) stock in experiments in which CP was the transposase source; however, it was not impaired in either type of male in an experiment in which \(\Delta2-3\) was the transposase source. Previous analyses failed to detect a maternal effect of either piwi\(^{d}\) or piwi\(^{\delta}\) on CP-induced P excisions from the sn\(^{aw}\) allele in the male germ line (Simmons et al., 2007). However, a recent study found that piwi\(^{d}\) disrupted TP5-mediated repression of dysgenic sterility in the daughters of females heterozygous for the mutation, although it did not disrupt repression of dysgenesis by another telomeric P element, TP6 (Belinco et al., 2009); by contrast, piwi\(^{\delta}\) had no effect on repression by either telomeric P element. In a related vein, Josse et al. (2007) found that females heterozygous for piwi\(^{d}\) or piwi\(^{\delta}\) had a reduced capacity for a telomere transsilencing effect (TSE); however, this reduction was seen only when the females were also heterozygous for the Su(var)205\(^{d}\) mutation. All these genetic data suggest that impairment of P-element regulation by a mutation in the piwi gene may depend on the nature of the mutation, the transposase source, the telomeric P element, the genetic assay and the genotype – for example, whether other compromising mutations such as Su(var)205\(^{d}\) are present. It should be noted that although both piwi mutations are due to transgene insertions, the insertion in piwi\(^{d}\) is expected to be more severe. This insertion is in the first coding exon, whereas the insertion in piwi\(^{\delta}\) is in the fourth coding exon (Cox et al., 1998); piwi\(^{\delta}\) may therefore encode a
Maternal impairment of P-element regulation

partially functional polypeptide, which may explain
why it has a less severe mutant phenotype (Lin &
Spradling, 1997) and why it does not impair P regu-
lation.

The RT–PCR analyses reported here indicate that
both TP5 and CP mRNAs accumulate in the germ
lines of females heterozygous for piwi\(^2\), but not in the
germ lines of females heterozygous for piwi\(^2\). The
processing of P-element mRNAs into piRNAs there-
fore appears to be impaired in the piwi\(^2\) heterozygotes
but not in the piwi\(^2\) heterozygotes. This finding par-
allels the observation that P-element regulation is
compromised in the sons of piwi\(^2\) heterozygotes but
not in the sons of piwi\(^2\) heterozygotes. It is not clear
what role the Piwi protein plays in the piRNA path-
way. Piwi is a nuclear protein that interacts physically
with HP1 (Brower-Toland et al., 2007). The Piwi
protein also binds piRNAs (Brennecke et al., 2007).
One possibility is that piRNA–Piwi complexes are
involved in the transcriptional repression of transpo-
sons, including some P elements (Yin & Lin, 2007).

Su(var)205 was the first gene to be implicated in
P-element regulation, and several reports have since
confirmed its relevance using different assays
(Ronsseray et al., 1996, 1998; Marin et al., 2000;
Haley et al., 2005; Josse et al., 2007; Belinco et al.,
2009). HP1, the protein encoded by this gene, is
associated with chromatin, especially heterochromatin
(James et al., 1989). In addition to organizing
chromatin, HP1 appears to perform a capping func-
tion at the ends of chromosomes, including XL
(Fanti et al., 1998); it is also present in the telomeric
retrotransposon array and in the TAS (Capkova
Frydrykova et al., 2008). When HP1 is depleted, as in
stocks that are heterozygous for a Su(var)205 mu-
tation, the telomeric capping function is compromised
and the chromosomes develop elongated retro-
transposon arrays (Savitsky et al., 2002). This genetic
change may contribute to the impairment of TP5-
mediated regulation of P element activity that is ob-
served in such stocks (Haley et al., 2005).

The RT–PCR experiments indicate that females
heterozygous for TP5 and the Su(var)205\(^4\) mutation
produce more germ-line TP5 mRNA than control
females lacking the mutation. However, given the
telomere-capping and chromatin-organizing role of
HP1, the increase in germ-line TP5 mRNA in females
heterozygous for the Su(var)205\(^4\) mutation is not
likely due to a malfunction in the ping-pong cycle
of the piRNA pathway, as it probably is in females
heterozygous for an aub mutation. Rather, the in-
crease might stem from more vigorous sensadow
transcription of the TP5 element. Elongated telomeric
retrotransposon arrays of the sort that develop in
stocks with Su(var)205 mutations are known to in-
crease the expression of elements inserted in the TAS
(Golubovsky et al., 2001). This increased expression
could result from stimulation of the TP5 promoter by
enhancers within the elongated retrotransposon
array, or from read-through transcription origin-
ing in the retrotransposon array. Alternately,
the increased senseward transcription of TP5 in
Su(var)205\(^4\)/+ females could simply be due to the de-
pletion of HP1 within the TAS (see Capkova
Frydrykova et al., 2008).

One indication that the ping-pong cycle of the
piRNA pathway is not disrupted by the Su(var)205\(^4\)
mutation is that the CP mRNA level is low in the
germ lines of females heterozygous for this mutation.
The RT–PCR analysis (Fig. 3) shows that this mRNA
level is consistently lower than the levels in TP5 w/w;
CP/mutation females heterozygous for the aub\(^{OC42}\),
aub\(^{OC4\,\,P-\,3\,\,u}\), or piwi\(^2\) alleles, which all impaired P regu-
lation, and that it is as low or lower than the level in
w/w; CP/+ control females, which cannot carry out
the piRNA pathway for P regulation because they
lack the critical TP5 element. The comparative dearth
of germ-line CP mRNA in TP5 w/w; CP/Su(var)205\(^4\)
females implies that this mRNA is being processed
successfully by the ping-pong cycle to feed the
piRNA pathway. Impairment of P regulation by the
Su(var)205\(^4\) mutation must therefore be due to a
breakdown in some other process. There is, by the
way, no dearth of somatic CP mRNA in females
carrying the Su(var)205\(^4\) mutation (Fig. 2). Thus,
the reduction in CP mRNA is limited to the germ line,
where the proteins thought to be involved in the
ping-pong cycle – Aub and Ago3 – are expressed
(Brennecke et al., 2007, 2008; Li et al., 2009; Tushir
et al., 2009), and where P excisions are regulated by
telomeric P elements such as TP5. Note also that the
level of germ-line CP mRNA in females carrying
Su(var)205\(^4\) is about the same as that in females car-
rying the piwi\(^2\) allele (Fig. 3), which has not been
found to impair P regulation by any assay. Thus,
the ping-pong cycle seems to be functioning as well
in TP5 w/w; CP/Su(var)205\(^4\) females, which carry a
proven disrupter of P regulation, as in TP5 w/w; CP/
piwi\(^2\) females, which do not.

If the ping-pong cycle is not impaired by
Su(var)205\(^4\), then what is the reason for this mu-
tation’s profoundly negative effect on P-element regu-
lation? Josse et al. (2007) have proposed that the
regulation by telomeric elements and transgenes has
both post-transcriptional and chromatin-organizing
aspects that depend on small RNAs generated from
the telomere. According to this proposal, antisense
piRNAs derived from a telomeric P element would be
the key ingredients in the post-transcriptional aspect
of P regulation because by targeting and destroying
P mRNAs, they would minimize the synthesis of the
P transposase, which is the agent of P-element move-
ment. However, by itself this destruction is apparently
not sufficient for effective repression of P-element
activity. Transposase-encoding mRNA is at the same low level in TP5 w/w; CP/Su(var)205* females as it is in TP5 w/w; CP/piw¹ females (Fig. 3); yet, the Su(var)205* mutation profoundly impairs P regulation through a maternal effect whereas the piw¹ mutation does not. Effective regulation of P-element activity must therefore involve something more than the destruction of transposase-encoding mRNA. Josse et al. (2007) suggest that the additional component is chromatin re-organization. Sense piRNAs produced by the destruction of mRNAs may be feed into the ping-pong cycle to generate a population of antisense piRNAs that subsequently associate with proteins such as Piwi to form complexes that bind to P elements throughout the genome. Such complexes may then re-organize the chromatin locally into a state that prevents the P transposase from catalysing P-element movement. Alternately, the piRNA–protein complexes could facilitate the transfer of a pre-existing repressive state from the telomere to other P elements. This process would require ectopic pairing between the telomeric and non-telomeric P elements. In either scenario, HP1 is likely to be involved because it is a known partner of Piwi and it is present in the telomere. The impairment of P regulation that occurs in flies with mutant Su(var)205 mothers could therefore reflect a breakdown in the ability to form piRNA–protein complexes that effectively re-organize chromatin into a repressed state. HP1-like proteins acting in concert with protein complexes that contain small RNAs have also been implicated in chromatin reorganization in Schizosaccharomyces pombe (Verdel et al., 2004; Grewal, 2010).

How does this hypothesized chromatin re-organization lead to a repression of P-element movement? One possibility is that HP1 binds to complete P elements and blocks, or significantly attenuates, the synthesis of mRNAs encoding the P transposase. In S. pombe, the end result of RNAi/HP1-mediated chromatin reorganization is transcriptional repression. However, our data indicate that the depletion of HP1 does not result in robust synthesis of transposase-encoding P mRNAs, as would be expected in this model; in fact, germ-line CP mRNA levels remain low in females that are heterozygous for an HP1-depleting mutation. This observation suggests that the regulation of P-element movement may involve a subtler mechanism. Instead of quashing P transcription, HP1-binding may simply prevent the P transposase from acting on its substrates – that is, it restricts the action of the P transposase but not that of the RNA polymerase. In this vein, it should be noted that a model of transcriptional repression implies that the loci into which P elements have been inserted run the risk of being silenced, and for a fly carrying 50–80 P elements in its diploid genome, this much of repression might be an unacceptable level of collateral damage.

The model of P regulation that emerges from all these considerations comprises four main steps. First, in the female germ line, piRNAs are generated from a telomeric P element by an as yet unknown mechanism. Second, these piRNAs are amplified by the ping-pong cycle – a process that involves the targeted destruction of P mRNAs, including the mRNA that encodes the P transposase. The Aub and Ago3 proteins carry out this process. Third, the piRNAs created by ping-pong cycling are transmitted to the offspring through the egg cytoplasm. Fourth, some of these piRNAs form complexes with proteins such as Piwi and HP1 to re-organize chromatin into a state that represses P-element movement. Piwi may act as a mediator between the ping-pong cycle and the chromatin re-organizing events by collecting piRNAs from Aub and guiding them to P elements throughout the genome, whereupon HP1 joins Piwi to foster a repressive chromatin state. In this model, HP1 is hypothesized to act downstream of Aub. However, recent analyses of the telomeric TSE have suggested that HP1 may also be involved in the production of piRNAs, possibly by stimulating the expression of their precursors from the TAS (Todeschini et al., 2010). This chromatin-organizing protein may therefore act upstream as well as downstream of Aub in the system that regulates P elements.

This work was supported by the Department of Genetics, Cell Biology and Development of the University of Minnesota, Benjamin Berres, Eric Chapman and Wojciech Kraszkiewicz helped to develop the P-transgene excision assay used in the genetic analyses.

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