Maternal enhancement of cytotype regulation in *Drosophila melanogaster* by genetic interactions between telomeric *P* elements and non-telomeric transgenic *P* elements

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(Received 13 August 2012; revised 30 October 2012)

Summary

The X-linked telomeric *P* elements (TPs) TP5 and TP6 regulate the activity of the entire *P* element family because they are inserted in a major locus for the production of Piwi-interacting RNAs (piRNAs). The potential for this cytotype regulation is significantly strengthened when either TP5 or TP6 is combined with a non-telomeric X-linked or autosomal transgene that contains a *P* element. By themselves, none of the transgenic *P* elements have any regulatory ability. Synergism between the telomeric and transgenic *P* elements is much greater when the TP is derived from a female. Once an enhanced regulatory state is established in a female, it is transmitted to her offspring independently of either the telomeric or transgenic *P* elements – that is, it works through a strictly maternal effect. Synergistic regulation collapses when either the telomeric or the transgenic *P* element is removed from the maternal genotype, and it is significantly impaired when the TPs come from stocks heterozygous for mutations in the genes *aubergine*, *piwi* or *Su(var)205*. The synergism between telomeric and transgenic *P* elements is consistent with a model in which *P* piRNAs are amplified by alternating, or ping-pong, targeting of primary piRNAs to sense and antisense *P* transcripts, with the sense transcripts being derived from the transgenic *P* element and the antisense transcripts being derived from the TP.

1. Introduction

Transposons are important components of the genomes of many organisms. Their activity causes mutations and chromosome breakage – damage that is best studied in model genetic organisms such as *Drosophila melanogaster*. Recent genetic and molecular analyses have revealed that *Drosophila* has elaborate mechanisms to repress transposon activity, and that small RNAs play key roles in some of these mechanisms (Josse *et al.*, 2007; Chambeyron *et al.*, 2008; Jensen *et al.*, 2008; Brennecke *et al.*, 2007, 2008; Klattenhoff *et al.*, 2009; Li *et al.*, 2009; Tushir *et al.*, 2009). The RNAs that interact with the Piwi class of proteins, called Piwi-interacting RNAs (piRNAs), appear to be especially important.

One of the major loci for the production of piRNAs is situated in the Telomere Associated Sequences (TAS) at the left end of the X chromosome. A transposon inserted in this locus generates both sense and antisense piRNAs (Brennecke *et al.*, 2008). The antisense piRNAs are of particular significance because they can be targeted to sense mRNAs produced by other copies of the transposon elsewhere in the genome. These mRNAs can then be cleaved into small fragments that become sense piRNAs, which may subsequently be targeted to antisense RNAs transcribed from the telomeric locus to generate more antisense piRNAs. With repetition, this alternating, or ping-pong, cycle is expected to produce a large population of sense and antisense piRNAs (Aravin *et al.*, 2007; Gunawardane *et al.*, 2007; Brennecke *et al.*, 2007, 2008; Li *et al.*, 2009) and, concomitantly, to destroy transposon mRNA (Jensen *et al.*, 2008). It has been speculated that some of the piRNAs may also guide transcription-inhibiting proteins to transposon copies present in the genome (Josse *et al.*, 2007; Simmons *et al.*, 2010). Thus, either by destroying transposon mRNA or by preventing its synthesis, piRNAs can undercut transposon expression and repress transposition.
Both retrotransposons and cut-and-paste transposons can be regulated by the piRNAs generated from ping-pong cycling. As a class, the retrotransposons are more numerous and may have a greater genetic and evolutionary significance. However, one cut-and-paste transposon, Drosophila’s P element, provides an unusual opportunity to study the ping-pong model of piRNA amplification because this element can be genetically manipulated in crosses.

P elements appear to have entered the genome of Drosophila melanogaster by horizontal transfer during the 20th century, and have since spread worldwide (Kidwell, 1983). P transposition is catalysed by a transposase encoded by complete members of the P element family (Karess & Rubin, 1984); incomplete P elements are unable to make the transposase, but they can be mobilized if the transposase is provided by a complete element somewhere in the genome. P-element movement occurs only in the germ line because transposase synthesis is restricted to that tissue (Laski et al., 1986). Within the germ line, P movement is regulated by a maternally transmitted condition called the P cytotype, which depends on the P elements themselves (Engels, 1979). Genetic analyses have revealed that this condition can be established by P elements inserted in the TAS of the XL telomere (Ronsseray et al., 1991, 1993, 1996, 1998; Marin et al., 2000; Stuart et al., 2002; Simmons et al., 2004; Niemi et al., 2004; Josse et al., 2007; Jensen et al., 2008), and molecular analyses with some of these elements have shown that they produce piRNAs (Brennecke et al., 2008). Cytotype regulation of the P-element family therefore appears to be mediated by maternally transmitted piRNAs (Brennecke et al., 2008; Thorp et al., 2009). Genetic analyses have also revealed that cytotype regulation anchored in a telomeric P element (TP) can be enhanced by numerous non-TPs scattered about the genome even though the latter have no intrinsic regulatory ability (Simmons et al., 2007). This synergism has been postulated to result from a ping-pong cycle fed by antisense RNAs from the TP and sense mRNAs from the other P elements (Belinco et al., 2009).

In the laboratory P elements can be activated by crossing them into strains that lack the P cytotype. These strains have the M cytotype, a condition that permits P-element movement. The M cytotype is characteristic of strains that do not have P elements in their genomes (pure M strains) and of some strains that have them; these latter strains are denoted M+. When strains with potentially active P elements (P strains) are crossed with M cytotype strains, the offspring may exhibit a syndrome of germ-line abnormalities called hybrid dysgenesis (Kidwell et al., 1977). This syndrome includes traits such as a high mutation rate, frequent chromosome breakage and sterility. The sterility occurs because the germ-line cells in the gonads are wiped out (Khurana et al., 2011). This phenomenon, called gonadal dysgenesis (GD), is enhanced by culturing the developing flies at 29 °C. Usually only the offspring from crosses between P males and M females exhibit dysgenesis. The offspring from the reciprocal cross, P females × M males, are not dysgenic because the P cytotype is transmitted maternally (Engels, 1979).

In this paper, we address several questions about cytotype regulation of the P-element family. Can this regulation be enhanced by introducing just one additional P element into a genotype that has an X-linked TP, and if so, what kinds of additional P elements can bring about the enhanced regulatory state? Can the additional enhancing element be on any of the major chromosomes? Can the enhanced regulatory state be transmitted to offspring independently of either of the two interacting P elements? Does it persist in subsequent generations when these elements are removed from the genotype? Is it sensitive to cytotype-disrupting mutations? Does the ping-pong cycle of piRNA formation provide an adequate explanation for the synergism between TPs and non-TPs. Can this synergism be explained by other molecular mechanisms?

2. Materials and methods

(i) Drosophila stocks and husbandry

Information on the mutant alleles used in the experiments is available on the Flybase website, in Lindsley & Zimm (1992), or in references cited in the text. The isolation and initial analysis of the TPs, TP5 and TP6 are described in Stuart et al. (2002). All stocks carrying these elements were marked with a wild-type allele of the yellow body locus (y+) and a mutant allele of the white eye locus (w) – both tightly linked to the XL telomere. Genomic Southern blotting and PCR with P-specific primers established that no other P elements were present in these stocks. TP5 strains that were heterozygous for mutations in the genes aubergine (aub), piwi and Suppressor of variegation 205 [Su(var)205], and correlated strains from which these mutations had been removed, are described in Belinco et al. (2009). Maps of TP5, TP6 and the other P elements used in this study are presented in Fig. 1.

Experimental cultures were reared in vials on a standard cornmeal-molasses-dried yeast medium at 25 °C unless otherwise specified; stock cultures were maintained in vials or in half-pint milk bottles at 18–21 °C.

(ii) Hobo transgenes with P-element sequences

Stocks carrying hobo transgenes with different terminally truncated (and therefore intrinsically immobile)
Fig. 1. Structures of \( P \) elements used in this study. The 31 bp inverted terminal repeats are represented by arrows. Exons are open boxes and introns are lines connecting the boxes. Missing sequences are indicated by dotted lines. The first or last nucleotides in particular segments of the elements are noted with reference to the nucleotides in the 2907 bp-long canonical complete \( P \) element, \( CP \). In the \( H(hsp/P) \) transgenes, the \( P \) element was truncated at nucleotide 38 and at either nucleotide 2688 (for \( P = TP5, TP6 \) or nucleotide 2872 (for \( P = CP, SP \)). The \( P^* \) element is a special case. This frameshifted \( P \) coding sequence spans nucleotides 153–2706 minus the introns; the frameshift is due to deletion of nucleotide 279. Recombination mapping established the genetic positions of the \( H(hsp/P) \) transgenes used in this study: \( H(hsp/CP)2 \) (2–9 2 or 34 8; located 12 8 cM from \( Sp \)), \( H(hsp/TP5)D \) (2–73 6), \( H(hsp/TP5)X \) (1–9 5), \( H(hsp/TP6)C \) (3–88 2), \( H(hsp/P^*) \) (3–0 3).

\( P \) elements situated downstream from the \( hsp70 \) promoter have been described (Simmons et al., 2002a, 2002b; Jensen et al., 2008). These transgenes, denoted in general as \( H(hsp/P) \), were inserted on the X chromosome, chromosome 2 or chromosome 3. The insertions were obtained by injecting embryos from an \( M \) strain that had been established to be free of \( P \) elements by PCR using \( P \)-specific primers. This strain has been characterized as \( E \) in the hobo system of hybrid dysgenesis – i.e. devoid of hobo transposase activity and unable to repress hobo transposition induced by crosses with \( H \) strains. A plasmid encoding the hobo transposase was co-injected with the \( hsp \) elements by PCR using \( P \)-specific primers. This \( P \*- \) PCR product was cut from its plasmid and inserted into the \( BamHI \) site downstream of the \( hsp70 \) promoter in the plasmid pMartini. Thirdly, the \( P^* \) PCR product was excised from pMartini using the restriction enzyme NotI, which recognizes sites on either side of the cassette, and the resulting NotI fragment was inserted into the unique NotI site in the hobo transformation vector pHawN (Blackman et al., 1989; Calvi & Gelbart, 1993), which contains the mini-white marker. The \( H(hsp/P^*) \) construct was then introduced into mutant \( w \) Drosophila by germ-line transformation using a plasmid source of the hobo transposase. The single insertion that was obtained, denoted \( H(hsp/P^*)B \), was localized to chromosome 3 and made homozygous by inbreeding. Genetic map positions of all the transgenes used in this study are given in the legend to Fig. 1.

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

RNA was isolated from groups of 20 females using TRIZOL (Invitrogen) according to the supplier’s instructions. The RNA was reverse transcribed into cDNA using the ThermoScript reverse transcriptase (Invitrogen) and a \( P \)-specific primer denoted P2575-u (5’-CAACATCGACGTTTCGCGCTG-3’), directed towards the 5’ end of the \( P \) element. After adding the primers \( P\Delta 0/1\)-d and \( P\Delta 2/3\)-u, the resulting cDNA was amplified by the PCR over 30 cycles using an appropriate temperature profile (see Jensen et al., 2008),
and the products were analysed in a 1% agarose gel by electrophoresis at 70 V.

(iv) Assay for GD

Repression of P-element movement is conveniently assayed by scoring females for the inability to produce eggs. This form of sterility, called gonadal dysgenesis (GD), is due to P-induced destruction of the germ-line cells (Nikki & Chigusa, 1986). To assay for GD, we squashed samples of females between two glass slides and looked for eggs. A solution of green food colouring helped to visualize the eggs extruded from each female. Any female that did not extrude eggs was scored as dysgenic.

The flies to be scored were produced by crossing females of a test genotype to males from either the Harwich \( w \) (Kidwell et al., 1977) or the Harwich \( y \ w P \) strains, which are both powerful inducers of GD in crosses to females from M strains. The Harwich \( y \ w \) strain was created by introducing the \( y \) and \( w^{77}c^{33} \) markers into the Harwich \( w \) stock. The test females were initially mass mated at 21 °C. After 3 days, they were aspirated into separate cultures, which were incubated at 29 °C. On day 11, all the offspring were transferred to a holding vial, where they matured for 2 days. As many as 20 females of each segregating genotype were then scored for GD.

(v) Statistical analyses

The frequency of GD was calculated independently for each class of offspring in each vial. Unweighted average frequencies and empirical standard errors \((se)\) among all the vials in a test group were then computed for each class. Averages and \(se\) for each group were also computed by pooling the raw data across classes in vials. Statistical differences between averages were evaluated by performing \(t\) or \(z\) tests.

3. Results

(i) Synergistic repression of P-element activity by combinations of telomeric and non-telomeric transgenic P elements

Although cytotype regulation is anchored in the TPs, it is enhanced by other \( P \) elements from M’ strains such as M5 Birmingham (Simmons et al., 2007). These strains contain numerous \( P \) elements that collectively have little or no regulatory ability. The stronger regulation that occurs in \( TP-M’ \) combinations therefore indicates that the telomeric and M’ elements interact synergistically. A single additional \( P \) element might be able to bring about this effect. To investigate this possibility, we tested individual transgenic \( P \) elements for interactions with the telomeric elements \( TP5 \) and \( TP6 \).

The test system involved flies that carried a \( TP \) on the X chromosome and a \( P \)-containing transgene inserted at a non-telomeric location on an autosome. The transgene was designed to express the \( P \) element from either of two promoters – the native \( P \) promoter, or the heat-shock-inducible \( hsp70 \) promoter, which was situated immediately upstream. However, in the experiments reported here, no heat shock treatments were employed. Each transgene was constructed using a \( hobo \) transformation vector (symbolized \( H \)) marked with the mini-white gene. Different \( P \) elements were inserted behind the \( hsp70 \) promoter within the \( hobo \) element to create four \( H(hsp/P) \) transgenes. RT–PCR analysis with the \( H(hsp/CP) \), \( H(hsp/TP5) \) and \( H(hsp/TP6) \) transgenes has shown that each of them produces \( P \) mRNA in the female germ line (Jensen et al., 2008), which is the physiologically relevant tissue for studies of \( P \)-element regulation. However, by themselves, neither these transgenes nor the \( H(hsp/SP) \) transgene has any ability to repress GD (Simmons et al., 2002b; Jensen et al., 2008). These transgenes were mapped by recombination with dominant markers; none of them proved to be near the telomerases or centromeres of chromosomes 2 or 3.

Interactions between the telomeric and transgenic \( P \) elements were assayed by scoring the daughters of \( TP y^+ w^+ y \ w; H(hsp/P)+ \) females for GD, which was induced by crossing these females to Harwich \( y \ w \) males. In these crosses, we could track the inheritance of the TP and the \( H(hsp/P) \) transgene by following the body and eye colour markers. Daughters with wild-type body colour carried the TP (which was tightly linked to the \( y^+ \) allele) and daughters with pigmented eyes carried the \( H(hsp/P) \) transgene (which contained the pigment-producing mini-white gene). This design allowed us to determine if synergistic repression of GD involved maternal or zygotic effects of the telomeric and transgenic \( P \) elements. In addition, we produced the \( TP y^+ w^+ y \ w; H(hsp/P)+ \) females for the test matings by performing reciprocal crosses between \( TP y^+ w \) flies and \( y \ w; H(hsp/P) \) flies (\( TP y^+ w \) as female in cross A and as male in cross B). This feature allowed us to determine if the parental origin of the TP and the \( H(hsp/P) \) transgene mattered.

Table 1 summarizes the results of the tests for synergistic regulation involving the telomeric element \( TP5 \). In the absence of any transgene, \( TP5 \) repressed GD moderately, but only in the daughters of the tested females from cross A. We observed 84% GD when \( TP5 \) was present in these daughters and 87% GD when it was absent. In comparison, we observed 98% GD in both classes of daughters from the tested females from cross B. The results from cross A and cross B are significantly different. Thus, as previously reported (Belinco et al., 2009; Thorp et al., 2009), in cross A the telomeric \( TP5 \) element moderately represses...
Table 1. Synergism between the telomeric TP5 element and various H(hsp/P) transgenes assessed in the F₂ daughters of TP5 y⁺ w/y w, H(hsp/P)/ + F₁ females from reciprocal crosses between TP5 y⁺ w and y w, H(hsp/P) strains

<table>
<thead>
<tr>
<th>Transgene^a</th>
<th>Cross^c</th>
<th>Neither^a</th>
<th>Transgene only^a</th>
<th>TP5 only^a</th>
<th>Both^a</th>
<th>Pooled overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>A</td>
<td>32</td>
<td>499</td>
<td>87.3 ± 2.3</td>
<td>167</td>
<td>88.5 ± 4.2</td>
</tr>
<tr>
<td>H(hsp/SP2)A</td>
<td>A</td>
<td>28</td>
<td>186</td>
<td>89.2 ± 2.1</td>
<td>157</td>
<td>21.3 ± 4.2</td>
</tr>
<tr>
<td>H(hsp/CP2)</td>
<td>A</td>
<td>30</td>
<td>147</td>
<td>82.4 ± 4.3</td>
<td>185</td>
<td>10.1 ± 3.6</td>
</tr>
<tr>
<td>H(hsp/TP55D</td>
<td>A</td>
<td>27</td>
<td>290</td>
<td>9.6 ± 3.1</td>
<td>272</td>
<td>55.1 ± 4.9</td>
</tr>
<tr>
<td>H(hsp/TP56C</td>
<td>A</td>
<td>32</td>
<td>290</td>
<td>55.9 ± 5.1</td>
<td>296</td>
<td>49.9 ± 5.2</td>
</tr>
<tr>
<td>None</td>
<td>B</td>
<td>29</td>
<td>266</td>
<td>98.6 ± 0.8</td>
<td>170</td>
<td>99.0 ± 1.0</td>
</tr>
<tr>
<td>H(hsp/SP2)B</td>
<td>B</td>
<td>25</td>
<td>150</td>
<td>99.0 ± 1.0</td>
<td>157</td>
<td>97.5 ± 1.5</td>
</tr>
<tr>
<td>H(hsp/CP2)B</td>
<td>B</td>
<td>23</td>
<td>95</td>
<td>94.3 ± 2.8</td>
<td>94</td>
<td>78.6 ± 4.6</td>
</tr>
<tr>
<td>H(hsp/TP55D</td>
<td>B</td>
<td>24</td>
<td>150</td>
<td>81.1 ± 4.3</td>
<td>133</td>
<td>81.6 ± 4.6</td>
</tr>
<tr>
<td>H(hsp/TP66C</td>
<td>B</td>
<td>7</td>
<td>61</td>
<td>98.6 ± 1.4</td>
<td>62</td>
<td>100.0</td>
</tr>
</tbody>
</table>

^a Two factors – the telomeric element TP5 and the H(hsp/P) transgene – segregated in the test crosses, giving rise to four genotypic classes in the F₂. The headings indicate which of these two factors were present in the females that were scored for GD.

^b The H(hsp/CP2) and H(hsp/TP55D) transgenes are located on chromosome 2 and the H(hsp/SP2) and H(hsp/TP66) transgenes are located on chromosome 3.

^c Cross A is TP5 y⁺ w females × y w, H(hsp/P) males, and cross B is TP5 y⁺ w males × y w, H(hsp/P) females.

<table>
<thead>
<tr>
<th>No. of flies</th>
<th>%GD ± se^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>999</td>
<td>85.9 ± 2.8</td>
</tr>
<tr>
<td>678</td>
<td>87.0 ± 2.0</td>
</tr>
<tr>
<td>664</td>
<td>20.0 ± 5.6</td>
</tr>
<tr>
<td>786</td>
<td>10.0 ± 3.1</td>
</tr>
<tr>
<td>1132</td>
<td>52.6 ± 4.5</td>
</tr>
<tr>
<td>511</td>
<td>98.6 ± 0.5</td>
</tr>
<tr>
<td>622</td>
<td>98.5 ± 0.6</td>
</tr>
<tr>
<td>427</td>
<td>84.8 ± 2.5</td>
</tr>
<tr>
<td>545</td>
<td>82.5 ± 2.6</td>
</tr>
<tr>
<td>232</td>
<td>98.8 ± 0.6</td>
</tr>
</tbody>
</table>

HD, however, it was not a repressor of GD in the F₂ females from cross A (69% GD, regardless of which or not they inherited the TP5 element to repress GD in the F₂). In each test, all four classes of F₂ daughters showed approximately equal frequencies of daughters segregated in these tests. There were roughly the same across all four classes. Thus, Table 1 adequately summarizes the data from each test.

Two factors – the telomeric element TP5 and the H(hsp/P) transgene – segregated in the test crosses, giving rise to four genotypic classes in the F₂. The headings indicate which of these two factors were present in the females that were scored for GD. The transgenes are located on chromosome 3. Cross A is TP5 y⁺ w females × y w, H(hsp/P) males, and cross B is TP5 y⁺ w males × y w, H(hsp/P) females.

The two telomeric elements TP5 and the H(hsp/P) transgene – segregated in the test crosses, giving rise to four genotypic classes in the F₂. The headings indicate which of these two factors were present in the females that were scored for GD. The transgenes are located on chromosome 3. Cross A is TP5 y⁺ w females × y w, H(hsp/P) males, and cross B is TP5 y⁺ w males × y w, H(hsp/P) females.

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In the various transgene combinations, TP5 interacted synergistically with H(hsp/CP2), H(hsp/CP2) and H(hsp/TP55C) to weaken the maternal effect. On this maternal effect, the transgenes were assessed in the F₂ daughters of TP5 y⁺ w and y w, H(hsp/P) strains. Table 1 adequately summarizes the data from each test.

HD, however, it was not a repressor of GD in the F₂ females from cross A (69% GD, regardless of which or not they inherited the TP5 element to repress GD in the F₂). In each test, all four classes of F₂ daughters showed approximately equal frequencies of daughters segregated in these tests. There were roughly the same across all four classes. Thus, Table 1 adequately summarizes the data from each test.
Table 2. Synergism between the telomeric TP6 element and various H(hsp/P) transgenes assessed in the F2 daughters of TP6 y+w/y w; H(hsp/P)/+ F1 females from reciprocal crosses between TP6 y+w and y w; H(hsp/P) strains.

<table>
<thead>
<tr>
<th>Transgene Cross</th>
<th>No. of vials</th>
<th>No. of flies</th>
<th>%GD ± se&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Transgene only&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of flies</th>
<th>%GD ± se&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TP6 only&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of flies</th>
<th>%GD ± se&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Both&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of flies</th>
<th>%GD ± se&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pooled overall</th>
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<tbody>
<tr>
<td>None A</td>
<td>29</td>
<td>386</td>
<td>69.3 ± 4.8</td>
<td>209</td>
<td>86.1 ± 3.7</td>
<td>197</td>
<td>90.2 ± 2.6</td>
<td>213</td>
<td>90.1 ± 3.3</td>
<td>772</td>
<td>69.3 ± 4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H(hsp/SP)A</td>
<td>30</td>
<td>217</td>
<td>88.1 ± 4.0</td>
<td>144</td>
<td>23.2 ± 5.6</td>
<td>146</td>
<td>27.8 ± 6.3</td>
<td>163</td>
<td>22.1 ± 6.4</td>
<td>836</td>
<td>87.9 ± 5.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H(hsp/CP)2</td>
<td>25</td>
<td>129</td>
<td>21.6 ± 6.2</td>
<td>241</td>
<td>5.8 ± 20</td>
<td>219</td>
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<td>H(hsp/TP5)D</td>
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<td>29.9 ± 3.4</td>
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<td></td>
</tr>
<tr>
<td>H(hsp/TP6)C</td>
<td>21</td>
<td>141</td>
<td>26.6 ± 42</td>
<td>228</td>
<td>98.9 ± 0.8</td>
<td>228</td>
<td>95.7 ± 1.7</td>
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<td>97.6 ± 1.0</td>
<td>467</td>
<td>94.8 ± 1.4</td>
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</tr>
<tr>
<td>None B</td>
<td>26</td>
<td>251</td>
<td>98.9 ± 0.8</td>
<td>101</td>
<td>97.2 ± 16</td>
<td>132</td>
<td>92.8 ± 2.3</td>
<td>115</td>
<td>92.7 ± 3.6</td>
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<td></td>
</tr>
<tr>
<td>H(hsp/SP)A B</td>
<td>22</td>
<td>119</td>
<td>99.4 ± 0.6</td>
<td>96</td>
<td>88.8 ± 3.2</td>
<td>116</td>
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</tr>
<tr>
<td>H(hsp/CP)2 B</td>
<td>22</td>
<td>108</td>
<td>80.3 ± 6.0</td>
<td>72</td>
<td>95.0 ± 2.5</td>
<td>91</td>
<td>90.5 ± 3.7</td>
<td>80</td>
<td>92.7 ± 3.0</td>
<td>332</td>
<td>89.2 ± 2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H(hsp/TP5)D B</td>
<td>20</td>
<td>89</td>
<td>81.7 ± 61</td>
<td>53</td>
<td>96.8 ± 21</td>
<td>57</td>
<td>100 ± 0</td>
<td>58</td>
<td>97.5 ± 2.5</td>
<td>226</td>
<td>98.1 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H(hsp/TP6)C B</td>
<td>8</td>
<td>58</td>
<td>93.8 ± 63</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Two factors – the telomeric element TP6 and the H(hsp/P) transgene – segregated in the test crosses, giving rise to four genotypic classes in the F2. The headings indicate which of these two factors were present in the females that were scored for GD.

<sup>b</sup> Cross A is TP6 y+w females × y w; H(hsp/P) males, and cross B is TP6 y+w males × y w; H(hsp/P) females.

<sup>c</sup> Unweighted average percentage GD ± se.
Table 3. Synergism between the telomeric TP5 element and the H(hsp/TP5)X transgene assessed in the F$_2$ daughters of TP5 y$^+$ w/y w H(hsp/TP5)X F$_1$ females from reciprocal crosses between TP5 y$^+$ w and y w H(hsp/TP5)X strains

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Cross</th>
<th>No. of vials</th>
<th>No. of flies</th>
<th>%GD ± se</th>
<th>No. of flies</th>
<th>%GD ± se</th>
<th>No. of flies</th>
<th>%GD ± se</th>
<th>No. of flies</th>
<th>%GD ± se</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>A</td>
<td>28</td>
<td>261</td>
<td>87.5 ± 2.6</td>
<td>300</td>
<td>87.6 ± 2.3</td>
<td>18</td>
<td>43.3 ± 13.0</td>
<td>561</td>
<td>87.0 ± 2.0</td>
</tr>
<tr>
<td>H(hsp/TP5)X</td>
<td>A</td>
<td>25</td>
<td>14</td>
<td>55.6 ± 14.4</td>
<td>238</td>
<td>51.6 ± 4.2</td>
<td>18</td>
<td>43.3 ± 13.0</td>
<td>572</td>
<td>53.4 ± 3.5</td>
</tr>
<tr>
<td>None</td>
<td>B</td>
<td>17</td>
<td>170</td>
<td>99.5 ± 0.5</td>
<td>180</td>
<td>99.5 ± 0.5</td>
<td>19</td>
<td>90.5 ± 7.4</td>
<td>703</td>
<td>97.9 ± 0.6</td>
</tr>
<tr>
<td>H(hsp/TP5)X</td>
<td>B</td>
<td>25</td>
<td>20</td>
<td>100 ± 0</td>
<td>325</td>
<td>97.0 ± 1.1</td>
<td>19</td>
<td>90.5 ± 7.4</td>
<td>703</td>
<td>97.9 ± 0.6</td>
</tr>
</tbody>
</table>

* Two X-linked factors – the telomeric element TP5 and the H(hsp/TP5)X transgene – segregated in the test crosses, giving rise to four genotypic classes in the F$_2$.

$^a$ Cross A is TP5 y$^+$ w females × F$^+$ males, and cross B is TP5 y$^+$ w males × y w H(hsp/TP5)X females.

$^b$ Unweighted average percentage GD ± se.

Enhancement of cytotype regulation through the X chromosome can be enhanced by the transgene, as shown in Table 3. This experiment shows that a different insertion of the TP5 element can enhance repression by the X-linked telomeric element. The results suggest that the enhanced repression is statistically significant, and that the effect is mediated by the transgene at non-telomeric locations on any of the major chromosomes in the D. melanogaster genome.
had no ability to repress GD. However, by themselves, both of the TPs were moderate repressors in cross A (83% GD with TP5 and 85% GD with TP6), but not in cross B (98 and 99% GD, respectively). In combination with the \( H(hsp/P^*)B \) transgene, repression by the TPs was enhanced significantly in cross A (20 and 25% GD), but not in cross B (97 and 98% GD). Thus, in cross A, the \( H(hsp/P^*)B \) transgene interacts synergistically with both the TPs to enhance cytotype regulation of the \( P \)-element family.

(iv) Collapse of synergistic repression when either the telomeric or transgenic \( P \) element is removed from the genotype

Stocks that contain TPs and many other dispersed \( P \) elements are powerful repressors of GD. When the TPs are removed from these stocks, repression ability persists, although much diminished, for at least one generation (Simmons et al., 2007). This lower level of repression has been explained by proposing that some of the other \( P \) elements are able to generate piRNAs, although much less vigorously than the major piRNA locus in the X telomere (Belinco et al., 2009). We tested whether or not repression could persist when a telomeric TP5 element is removed from a synergistic TP5-\( H(hsp/P) \) combination by measuring the repression ability of \( y^+ w; H(hsp/TP5)D/+ \) females derived from crosses between TP5 \( y^+ w/y w; H(hsp/TP5)D/+ \) mothers and \( y^+ w \) fathers. The results of these tests, which spanned two generations, are summarized in Table 5.

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{TP} & \text{Transgene} & \text{Cross} & \text{No. of vials} & \text{No. of flies} & \%GD \pm \text{se}^a \\
\hline
\text{None} & H(hsp/P^*)B & C(A) & 22 & 513 & 100 \pm 0 \\
\text{None} & H(hsp/P^*)B & C(B) & 19 & 463 & 99.8 \pm 0.2 \\
TP5 & None & A & 26 & 520 & 82.9 \pm 2.6 \\
TP5 & H(hsp/P^*)B & A & 27 & 646 & 19.6 \pm 3.1 \\
TP5 & None & B & 24 & 429 & 97.9 \pm 0.8 \\
TP5 & H(hsp/P^*)B & B & 20 & 620 & 97.2 \pm 1.1 \\
TP6 & None & A & 25 & 488 & 85.5 \pm 1.9 \\
TP6 & H(hsp/P^*)B & A & 25 & 493 & 25.2 \pm 3.7 \\
TP6 & None & B & 25 & 488 & 99.2 \pm 0.4 \\
TP6 & H(hsp/P^*)B & B & 21 & 482 & 98.0 \pm 1.0 \\
\hline
\end{array}
\]

The initial crosses in this experiment were TP \( y^+ w \) females \( \times y w \) males or \( y^+ w; H(hsp/P^*)B \) males (cross A) and \( y w \) females or \( y^+ w; H(hsp/P^*)B \) females \( \times TP \ y^+ w \) males (cross B). The \( F_1 \) females from these crosses were mated to Harwich \( y w \) males and their daughters were scored for GD. In the control crosses \( [C(A) \text{ and } C(B)] \), the \( y w \) females were substituted for the \( TP \) strain. Females with and without the \( H(hsp/P^*)B \) transgene were scored separately; however, because there were no significant differences between these two groups, the data have been pooled.

\( ^a \) Unweighted average percentage GD \( \pm \) se.

(v) Impairment of synergistic repression by mutations in \( aub \), \( piwi \) and \( Su(var)205 \)

The proteins encoded by the genes \( aub \) and \( piwi \) play important roles in the piRNA pathway (Brennecke et al., 2007; Tushir et al., 2009), and the protein encoded by the \( Suppressor of variegation 205 \) gene – known as heterochromatin protein 1 (HP1) – plays an important role in chromatin organization (James et al., 1989). Mutational depletion of any of these proteins can impair \( P \)-element regulation profoundly. In particular, TPs in stocks that have been kept heterozygous for some \( aub \), \( piwi \) or \( Su(var)205 \) mutations do not establish strong synergism with other \( P \) elements (Belinco et al., 2009). These same mutations would, therefore, be expected to prevent synergism between the telomeric TP5 element and the
Table 5. Collapse of synergistic repression of GD in the granddaughters of TP5 y+ w/y w; H(hsp/TP5)D/+ females

<table>
<thead>
<tr>
<th>Generation 2</th>
<th>Class</th>
<th>Genotype</th>
<th>No. of vials</th>
<th>No. of flies</th>
<th>GD + se*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP5 y+ w/y w; H(hsp/TP5)D/+</td>
<td>1</td>
<td>TP5 y+ w/y w; H(hsp/TP5)D/+</td>
<td>26</td>
<td>504</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>TP5 y+ w/y w</td>
<td>26</td>
<td>476</td>
<td>10.7 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>y w/y w; H(hsp/TP5)D/+</td>
<td>21</td>
<td>381</td>
<td>78.2 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>y w/y w</td>
<td>28</td>
<td>559</td>
<td>99.8 ± 0.1</td>
</tr>
<tr>
<td>TP5 y+ w/y w (control)</td>
<td></td>
<td></td>
<td>31</td>
<td>933</td>
<td>72.5 ± 4.4</td>
</tr>
</tbody>
</table>

The females of generation 1 were produced by crossing TP5 y+ w females with y w; H(hsp/TP5)D males and the females of generation 2 were produced by crossing generation 1 females with y w males. Tests for repression of GD were conducted by crossing samples of generation 1 or generation 2 females to Harwich y males. The data have been pooled over the genotypes that segregated in these crosses.

* Unweighted average percentage GD ± se.

H(hsp/TP5)D transgene. To test this prediction, we crossed the H(hsp/TP5)D transgene into TP5 stocks that were heterozygous for aub, piwi or Su(var)205 mutations and evaluated the resulting TP5 y+ w/y w; mutation; H(hsp/TP5)D females for their ability to repress GD in the next generation. We also evaluated TP5 y+ w/y w; +/H(hsp/TP5)D females from parallel crosses in which the various mutations had been removed from the TP5 stocks many generations earlier.

As a negative control in this experiment, we crossed the H(hsp/TP5)D transgene into an M strain that did not have any P elements. When 24 females from this control were test crossed to Harwich w males, 98.6 ± 0.7% of their 345 daughters were dysgenic. Thus, by itself, the H(hsp/TP5)D transgene could not repress GD. As a positive control, we crossed the H(hsp/TP5)D transgene into a TP5 stock that was heterozygous for Gla, a mutation that has not been implicated in any aspect of piRNA-mediated regulation; this stock was the source of the telomeric TP5 element in all the other mutant stocks. When 25 females from this control were test crossed to Harwich w males, 17.0 ± 2.1% of their 486 daughters were dysgenic. Thus, when H(hsp/TP5)D was combined with TP5 from the root Gla stock, GD was repressed strongly.

The results of the tests with the other mutant and mutant-free TP5 stocks are summarized in Table 6. In general, when H(hsp/TP5)D was crossed into the TP5 stocks from which the aub, piwi and Su(var)205 mutations had been removed, dysgenesis was repressed strongly (9.6–19.4% GD), as in the positive control. The only exception was the stock from which piwi2 had been removed, where the GD frequency was 52.4%. This higher frequency does not appear to be related to any long-term effect of the piwi2 mutation; rather, it may simply be due to a random change in the structure and properties of the XL telomere (Belinco et al., 2009).

When H(hsp/TP5)D was crossed into the TP5 stocks that were heterozygous for the various mutations, GD was generally not repressed strongly (42.7–85.4% GD). The only exception was the stock heterozygous for piwi2, where the GD frequency was 9.3%. This lower frequency may reflect that piwi2 is a weaker mutant allele than piwi1; for instance, in homozygous condition piwi2 causes female sterility, whereas homozygous piwi1 also causes male sterility. A comparison of the left and right sides of Table 6 indicates that synergism between TP5 and H(hsp/TP5)D was impaired when the TP5 element came from a stock that was heterozygous for aubC2P–31w, aubC2Q34, piwi1 or Su(var)205. These results are similar to those from tests for synergism between TP5 and an ensemble of non-TPs (Belinco et al., 2009). The potential for synergism between TP5 and another P element—for example, the one in the H(hsp/TP5)D transgene—therefore appears to be sensitive to the mutational depletion of proteins encoded by the aub, piwi and Su(var)205 genes. It is important to recognize that this conclusion is based on tests with TP5 stocks that were heterozygous for the various mutations, and that in these stocks the capacity for synergism may have been impaired by the long-term effects of the mutations on the function of the TP5 element within the XL telomere. However, this impairment is not permanent because when TP5 stocks from which the mutations were removed many generations earlier are tested for synergism with H(hsp/TP5)D, dysgenesis is once again repressed strongly. The negative effects of the mutations can therefore be reversed after the mutations have been removed from the stocks.

4. Discussion

P elements provide an unusual opportunity to elucidate the mechanisms that regulate eukaryotic transposons. Individual P elements can be isolated in an
otherwise P-element-free genotype and then assessed for their abilities to prevent hybrid dysgenesis in test crosses. The regulatory abilities of combinations of P elements can also be assessed. These experimental tests are specific for P-element activity in the germ line and yield quantitative data to document it. The experimental end-points – for example, the frequency of GD in the offspring of a test cross – therefore directly reveal whether or not particular P elements, or combinations of P elements, are able to prevent P excision and transposition in the germ line. No other transposon affords the possibility of defining and controlling the genotype so precisely, and of connecting it to quantitative data on transposition.

We have used genetic manipulations to determine if the regulatory abilities of TPs – which are anchors of the P cytotype – can be enhanced by other P elements at non-telomeric locations. The telomeric elements TP5 and TP6, both inserted in the TAS of chromosome XL, repress GD in their own right, presumably because they are situated in a major locus for the production of piRNAs. Other P elements in the XL TAS have been shown to repress GD (Ronsseray et al., 1991; Marin et al., 2000), and their piRNA output has been documented (Brennecke et al., 2008). Furthermore, these piRNAs are deposited maternally in eggs (Brennecke et al., 2008), which is consistent with the abilities of X-linked TPs to repress GD through strictly maternal effects. Maternally deposited small RNAs have also been implicated in the repression of dysgenesis induced by the Penelope transposon in D. virilis (Blumenstiel & Hartl, 2005).

We have shown that the regulatory abilities of TP5 and TP6 are markedly enhanced by different types of transgenic P elements inserted at non-telomeric locations on the X chromosome or on either of the major autosomes. In their own right, none of these transgenic P elements has any ability to repress GD. The enhancement of regulatory ability must therefore be due to synergism between the telomeric and transgenic P elements, not to the addition of separate regulatory effects. Previous work had shown that TP5 and TP6 repress dysgenesis synergistically when combined with an ensemble of heterogeneous, dispersed, non-TPs (Simmons et al., 2007; Belinco et al., 2009). We now know that the regulatory abilities of these TPs can be enhanced synergistically by a single transgenic P element.

Both small and large transgenic P elements enhanced the regulatory abilities of the TPs. The small enhancing elements were transgenic clones of TP5 and TP6. Each of these transgenic TPs was effective in boosting the regulatory ability of each of the native TPs. However, with both of the native TPs, the transgenic TP5 element was a more effective enhancer than the transgenic TP6 element. That the transgenic TP5 should be more effective when combined with its cognate telomeric TP5 is perhaps not surprising because these two elements are perfectly identical (except for the terminal truncations in the transgenic construct). However, the transgenic TP5 element was also a better enhancer in combination with the telomeric TP6 element, with which it shares only 83% of its sequence. Thus, regulatory enhancement is not simply a function of the amount of sequence shared by the telomeric and transgenic P elements. Other features of the elements, such as their expression level, their specific sequence composition, or the ease with which their RNA products are transported within and between cells, could be relevant. However, some minimum amount of shared sequence appears to be needed for synergism because the very small transgenic SP element did not enhance regulation when it was combined with either of the TPs.

The large transgenic P elements CP and P* both boosted regulation with each of the TPs. CP encodes the P transposase and might be expected to exacerbate dysgenesis. However, when combined with either TP, it led to substantially less dysgenesis in the test cross offspring. P* is a frame-shifted P coding sequence minus the native P promoter and all three P introns. When positioned downstream of the hsp70 promoter

---

**Table 6. Effects of aub, piwi and Su(var)205 mutations on synergism between the telomeric TP5 element and the H(hsp/TP5)D transgene**

<table>
<thead>
<tr>
<th>Mutation present in stock</th>
<th>Mutation removed from stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of vials</td>
<td>No. of flies</td>
</tr>
<tr>
<td>aub/+ P-aub</td>
<td>25</td>
</tr>
<tr>
<td>aub/dca</td>
<td>25</td>
</tr>
<tr>
<td>piwi1</td>
<td>25</td>
</tr>
<tr>
<td>piwi2</td>
<td>25</td>
</tr>
<tr>
<td>Su(var)2054</td>
<td>22</td>
</tr>
</tbody>
</table>

TP5 y+ w females from stocks with and without the listed mutations (Belinco et al., 2009) were crossed to y w; H(hsp/TP5)D males to produce TP5 y+ w/y w; (mutation) H(hsp/TP5)D females, which were then tested for repression of GD by crosses to Harwich w males. Daughters with different TP5 and H(hsp/TP5)D genotypes were not scored separately.

*(a) Unweighted average percentage GD ± se.*
in a *hobo* transgene, this element also enhanced *TP*-anchored regulation significantly. Thus, regulatory synergism occurs even when the transgenic *P* element encodes the transposase or when it lacks the native *P* promoter and all three *P* introns. It is interesting, however, that neither the *CP* nor *P*\(^*\) transgenic elements was as effective as the transgenic *TP5* element in boosting regulation. Both of the TPs share most of their sequence with these two transgenic *P* elements. Thus, as discussed above, shared sequence is not the sole determiner of enhanced regulation.

Previous studies have shown that a *TP*’s ability to repress GD is transmitted to test cross offspring as a strictly maternal effect – that is, offspring that do not inherit the *TP* repress GD as well as those that do inherit it (Thorp et al., 2009; Belinco et al., 2009; Simmons et al., 2010). This observation indicates (1) that repression involves a product of the *TP*, not the *TP* itself, (2) that the amount of product transmitted through the egg is sufficient to repress GD in the zygote, although perhaps not in every zygote and (3) that if any more *TP* product is synthesized in the zygote, it does not make repression any stronger. Thus, the final level of repression appears to be established – that is, set – in the maternal germ line. These conclusions also hold when repression is enhanced by combining a transgenic *P* element with a *TP* in the mother’s genotype. Test cross offspring that inherit neither transgenic *P* nor *TP* repress GD as well as offspring that inherit both. Cases in which the maternal genotype brings about strong, but incomplete, repression are particularly interesting. For example, when *H(hsp/CP)*\(^2\) is combined with either of the TPs the GD frequency in the test cross offspring is around 20%, regardless of the offspring’s genotype (Tables 1 and 2). Offspring that inherit the *TP*, *H(hsp/CP)*\(^2\), or both are not better at repressing GD than offspring that inherit neither of these factors even though there is clearly ‘room for improvement’. The enhanced regulatory state, like the basal regulatory state, therefore appears to be set in the maternal germ line.

One other feature of the experimental data is that the level of repression in the test cross offspring is strongly influenced by the grand-parental origin of the *TP*. When the *TP* is derived from the grandmother and the transgenic *P* element from the grandfather, repression is much stronger than when the derivation is reversed. *TPs* that are paternally derived completely lose their regulatory power (Stuart et al., 2002; Simmons et al., 2004). However, these elements can be ‘resuscitated’ if they pass through the germ line of a daughter (Niemi et al., 2004). From the data in Tables 1 and 2, it appears that resuscitation is facilitated by the presence of a transgenic *P* element in the daughter’s genotype.

What molecular mechanisms underlie these phenomena? Cytotype regulation appears to be mediated by piRNAs generated from *P* elements inserted in the TAS of chromosome XL (Brennecke et al., 2008). The biogenesis of these RNAs from the TPs is not understood. However, once formed, it is thought that the piRNA population is amplified by a ping-pong cycle fed by antisense RNAs transcribed from the *TP* and sense RNAs transcribed from other *P* elements (Brennecke et al., 2008; Belinco et al., 2009). Our data are consistent with this hypothesis. *P* elements contained within *hobo* transgenes clearly strengthen the regulatory abilities of TPs, presumably by providing the sense transcripts needed to amplify *P*-specific piRNAs in the maternal germ line. Ping-pong amplification of piRNAs is thought to occur in the nuage, a region on the cytoplasmic side of the nuclear membrane in germ line cells (Lim & Kai, 2007; Kibanov et al., 2011; Nagao et al., 2011; Zhang et al., 2011; Anand & Kai, 2012). Several proteins implicated in ping-pong cycling have been localized to the nuage. It is possible that *P*-element transcripts exported from germ-line nuclei could be processed into piRNAs by these proteins, particularly in the nurse cells, which could, in turn, export them to the developing oocyte where they would accumulate to provide a defence against *P* activity in the future embryo. Synergism between the telomeric and transgenic *P* elements is therefore consistent with an important role for ping-pong cycling in cytotype regulation.

Synergism might also be explained by transcription of *P* mRNAs by an RNA-dependent RNA polymerase (RdRP), generating antisense *P* RNAs that might feed into a pathway for the production of small interfering RNAs (siRNAs). However, we found that cytotype enhancement is impaired by mutations in the *aub* and *piwi* genes, both of which encode proteins that bind piRNAs. In addition, a telomeric *P* trans-silencing effect is impaired by mutations in these two genes, as well as by mutations in other genes known to be involved in the piRNA pathway; however, it is not impaired by mutations in *r2d2*, a gene in the siRNA pathway, or in *loquacious*, a gene in the miRNA and endo-siRNA pathways (Josse et al., 2007; Todeschini et al., 2010). These findings argue that cytotype regulation is mediated by piRNAs rather than siRNAs. Furthermore, there is currently no evidence for an RdRP in *Drosophila*. The amplification of piRNAs by ping-pong cycling therefore appears to be the more plausible explanation for how non-TPs enhance *TP*-anchored cytotype regulation in the *D. melanogaster* germ line.

Jordan Becker prepared Fig. 1 and Donald Rio provided a clone containing the frameshifted *P*-element coding sequence *P*\(^*\). Johng Lim made helpful comments on an early version of the manuscript and Justin Blumenstiel read the final manuscript and suggested some improvements. The experimentation was supported by funds from the Department of Genetics, Cell Biology, and Development of...
the University of Minnesota. Construction of the H(hsp70/P*) transgene was accomplished under the auspices of a past grant from the National Institutes of Health.

References


