Genetic Interactions Between $P$ Elements Involved in piRNA-Mediated Repression of Hybrid Dysgenesis in *Drosophila melanogaster*

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**ABSTRACT** Previous studies have shown that telomeric $P$ elements inserted at the left end of the X chromosome are anchors of the $P$ cytotype, the maternally inherited state that regulates $P$-element activity in the germ line of *Drosophila melanogaster*. This regulation is mediated by small RNAs that associate with the Piwi family of proteins (piRNAs). We extend the analysis of cytotype regulation by studying new combinations of telomeric and nontelomeric $P$ elements (TPs and non-TPs). TPs interact with each other to enhance cytotype regulation. This synergism involves a strictly maternal effect, called presetting, which is apparently mediated by piRNAs transmitted through the egg. Presetting by a maternal TP can elicit regulation by an inactive paternally inherited TP, possibly by stimulating its production of primary piRNAs. When one TP has come from a stock heterozygous for a mutation in the *aubergine*, *piwi*, or *Suppressor of variegation 205* genes, the synergism between two TPs is impaired. TPs also interact with non-TPs to enhance cytotype regulation, even though the non-TPs lack regulatory ability on their own. Non-TPs are not susceptible to presetting by a TP, nor is a TP susceptible to presetting by a non-TP. The synergism between TPs and non-TPs is stronger when the TP was inherited maternally. This synergism may be due to the accumulation of secondary piRNAs created by ping-pong cycling between primary piRNAs from the TPs and mRNAs from the non-TPs. Maternal transmission of $P$-element piRNAs plays an important role in the maintenance of strong cytotype regulation over generations.

Small RNAs that interact with the Piwi class of proteins—the piRNAs—have been implicated in the regulation of many different families of transposons in the genome of *Drosophila melanogaster* (Brennecke et al. 2007, 2008). Among these, the $P$-element family affords an opportunity to dissect this regulatory mechanism genetically and to evaluate it phenotypically. Flies carrying particular $P$ elements can be crossed to analyze the components of regulation and to study how these components contribute to the repression of harmful transposon activity. In this article, we investigate interactions between the transposons that anchor $P$-element regulation—located in the telomeric heterochromatin of the X chromosome—and interactions between these telomeric transposons and dispersed, nontelomeric transposons that can strengthen regulation.

$P$ elements are mobilized by a transposase encoded by structurally complete members of the $P$ family (Karess and Rubin 1984). Their activity is normally restricted to the germ line, where it causes hybrid dysgenesis, a syndrome of abnormalities that includes temperature-sensitive sterility and high frequencies of mutation and chromosome breakage (Kidwell et al. 1977). These traits occur in the offspring from crosses between $P$ (paternally contributing) males and M (maternally contributing) females, but they are rare or absent in the offspring from crosses between $P$ females and M males, or from $P \times P$ or $M \times M$ crosses. Flies from $P$ strains have $P$ elements in their genomes, but flies from $M$ strains typically do not; those that do are denoted as $M^\prime$ (Bingham et al. 1982). The low frequency of dysgenic traits in the offspring of crosses involving $P$ females indicates that $P$ elements are regulated by a maternally transmitted property of $P$ strains. This property, called the $P$ cytotype (Engels 1979), is mediated by $P$-element piRNAs (Brennecke et al. 2008).
Table 1 Gonadal dysgenesis in the daughters of females homozygous or heterozygous for a TP

| Females Homozygous for TP | TP Initially Derived from Females (Cross A) | Females Heterozygous for TP | TP Initially Derived from Males (Cross B) |
|---------------------------|------------------------------------------|-----------------------------|-----------------------------------------|
| TP                        | No. Vials | No. Flies | %GD ± SE  | No. Vials | No. Flies | %GD ± SE  | No. Vials | No. Flies | %GD ± SE  |
| None (Sam)                | 11        | 81        | 99.3 ± 0.7 | 30        | 576       | 99.3 ± 0.4 | 30        | 550       | 99.8 ± 0.2 |
| None (w)                  | 10        | 83        | 100        | 39        | 971       | 94.6 ± 0.9 | 33        | 783       | 98.2 ± 0.7 |
| TP5d                      | 28        | 373       | 13.7 ± 3.8 | 37        | 855       | 78.2 ± 2.2 | 34        | 769       | 96.8 ± 0.7 |
| TP6d                      | 27        | 281       | 32.5 ± 4.6 | 39        | 993       | 64.0 ± 2.7 | 32        | 678       | 99.0 ± 0.4 |
| NAe                       | 17        | 145       | 0          |           |           |           |           |           |           |

Gonadal dysgenesis was assessed in the daughters of test crosses between females homozygous or heterozygous for a TP and Harwich y w males. In crosses that segregated different genotypes, the daughters with the TP and those without it were scored separately, but because there were no differences between them, the results have been pooled.

- TP is tightly linked to the w allele of the w locus.
- NA is tightly linked to the w allele of the w locus.

One locus that produces piRNAs is situated within the telomere-associated sequences (TAS) at the end of the arm of the X chromosome—that is, at the telomere of XL (Brennecke et al. 2007). The TAS is an array of repeats with variable structure and length. Another array of repeats, distal to the TAS and forming the actual end of XL, consists of sequences derived from non-LTR retrotransposons (Mason and Biessmann 1995). Both of these arrays are associated with proteins, including heterochromatin protein 1 (HP1), which is the product of the suppressor of variegation 205 [Su(var)205] gene (James et al. 1989; Capkova Frydrykova et al. 2008). Piwi, the protein encoded by the piwi gene, may also be present (Brower-Toland et al. 2007; Yin and Lin 2007). The TAS locus produces both sense and antisense piRNAs that match sequences within its repeats; these types of piRNAs have therefore been called repeat-associated small interfering (rasi) RNAs (Vagin et al. 2006). If a P element has inserted into the TAS, then piRNAs consisting of sense and antisense P sequences are also produced (Brennecke et al. 2008). The TAS locus, with its inserted P element, therefore serves as an anchor of the P cytotype.

Cytotype regulation is established and maintained by TPs in the female germ line (Ronsseer et al. 1991; Marin et al. 2000; Stuart et al. 2002; Niemi et al. 2004; Simmons et al. 2004). Once established, a female can transmit the capacity for regulation to her daughters through the cytoplasm of her eggs—that is, as a strictly maternal effect of the anchoring TP (Ronsseer et al. 1993; Simmons et al. 2007a). This maternal effect implies that regulation is mediated by extrachromosomal factors, presumably piRNAs that were generated by the mother’s TP. However, this strictly maternal effect appears to be insufficient to repress P transposition in males (Stuart et al. 2002; Thorp et al. 2009). Cytotype regulation does occur in males, but only if they carry a maternally inherited TP. A TP that was inherited paternally, i.e., from father to son, as in crosses involving females with attached-X chromosomes, does not regulate P activity (Niemi et al. 2004; Simmons et al. 2004). When a male’s TP is transmitted to his daughters, as in crosses with females with unattached X chromosomes, its regulatory ability depends on the genotype of the male’s mate (Niemi et al. 2004). If the mate comes from an M strain that does not carry a TP, then the paternally inherited TP has little or no regulatory ability—that is, it is inactive. If the mate is heterozygous for a TP, then the paternally inherited TP can be activated by an extrachromosomal effect of the mate’s TP. This strictly maternal effect has been termed the “pre-P cytotype” (Ronsseer et al. 1993) or “presetting” (Niemi et al. 2004). Recent analyses suggest that this phenomenon is mediated by maternally inherited piRNAs, and that it is akin to parasitization in plants (De Vanssay et al. 2012). If the male’s mate also transmits a TP to her daughters, then this TP may enhance the reactivation of the paternally inherited TP (Niemi et al. 2004).

Cytotype regulation anchored in TPs can be strengthened by P elements at nontelomeric loci even though these non-TPs have no regulatory ability on their own (Belinco et al. 2009; Simmons et al. 2007a, 2012). This synergism is thought to result from a process involving RNAs from the two types of P elements. In brief, antisense piRNAs from the TPs target and cleave sense RNAs from the non-TPs to create a population of sense piRNAs, which in turn target and cleave antisense transcripts from the TPs to create more antisense piRNAs. With repetition, this process, called the ping-pong cycle (Aravin et al. 2007 Gunawardane et al. 2007; Brennecke et al. 2007, 2008; Li et al. 2009), amplifies the pool of P-element piRNAs so that cytotype regulation is strengthened. The enhanced regulatory ability is transmitted through eggs independently of the TPs and the non-TPs—that is, it is inherited as a strictly maternal effect (Simmons et al. 2012).

In this article, we extend the study of genetic interactions between different TPs, interactions between TPs and non-TPs, and how pre-setting affects these two types of interactions. Several questions are considered. Do two TPs interact synergistically to bring about strong cytotype regulation? Can a TP interact with or preset a TP that has a different DNA sequence? Is synergism between two TPs sensitive to mutational depletion of the proteins HP1, Piwi, or Aub [a cytoplasmic member of the Piwi family encoded by the aubergine (aub) gene]? How does synergism between TPs and non-TPs compare to synergism between TPs? Can a TP preset a non-TP, and can a non-TP preset a TP? To answer these questions, we focus on one aspect of dysgenesis, the temperature-sensitive sterility seen in the offspring of crosses between M females and P males. This trait, called gonadal dysgenesis (GD), is due to massive killing of the germ cells by rampant P-element activity (Nikki and Chigusa 1986; Khurana et al. 2011). Females that carry TPs are able to repress GD in at least some of their daughters. Accordingly, we use the frequency of GD to measure the strength of cytotype regulation; a low frequency implies strong regulation. Our analyses demonstrate the importance of genetic interactions between TPs and between TPs and non-TPs in the regulation of the P-transposon family.
is a null mutation causing bleach white eyes, and for canonical P to a mutant allele of the elements encodes the P transposase. These elements are all tightly linked to be brown. These mutant alleles make it possible to track the males of a strong P strain such as Harwich. Gonadal dysgenesis (GD) occurs when elements inherited from P strain and examined their daughters for gonadal dysgenesis; thus, Muller-5 Birmingham is a strain (Simmons et al. 2007a). Experimental cultures were incubated at 25°C. We calculated the percentage of females with GD in each vial and then computed the unweighted average percentage of GD for all the vials in the test group. The SE of this average was obtained from the associated empirical variance. Differences between averages were evaluated by performing t or z tests.

RESULTS

Synergism between homozygous telomeric P elements

Previous studies have not directly addressed if cytotype regulation is enhanced by synergistic interactions between two X-linked TPs. To investigate this issue, we performed test crosses between females that were homozygous or heterozygous for particular TPs and males from the Harwich y w P strain and examined their daughters for gonadal dysgenesis (Table 1). We also included test crosses with females from two M strains that did not carry a TP (or any other P element).

Almost all (>99%) of the daughters from the M females were dysgenic, demonstrating that Harwich y w is a powerful inducer of...
markers made it possible to track the inheritance of the various telomeric P elements throughout the experiment. All the crosses in this scheme were incubated at 25°C; however, test crosses between the various types of females and Harwich y w males were performed as described in the Materials and Methods.

GD. In contrast, most of the daughters of the TP homozygotes were not dysgenic, showing that they could repress the activity of the Harwich P elements. The NA and TP5 homozygotes were the strongest repressors, with 0% and 13.7% dysgenic daughters, respectively. The TP6 homozygotes, with 32.5% dysgenic daughters, repressed GD less strongly.

The TP heterozygotes that we studied came from two sets of crosses. In one set, homozygous TP females were mated to males from an M strain (cross A); in the other set, females from the M strain were mated to TP males (cross B)—that is, the A and B types of heterozygotes came from reciprocal crosses between the TP and M strains. These two types of heterozygotes were genetically (i.e., chromosomally) identical. However, they differed in the extrachromosomal factors that are transmitted through the egg cytoplasm. The cross B heterozygotes did not repress GD in their daughters, whereas the cross A heterozygotes did—a difference indicating that the paternally inherited TP in the cross B heterozygotes is inactive. Among the cross A heterozygotes, those carrying either TP6 or NA were moderate repressors (78.2% GD and 64.0% GD, respectively), whereas those carrying TP5 were very weak repressors (94.6% GD). Repression by the cross A heterozygotes was seen equally in the daughters that carried a TP and in those that did not. Thus, the repression was mediated by a strictly maternal (i.e., extrachromosomal) effect. None of the TP heterozygotes from cross A repressed GD as well as the TP homozygotes did. Doubling the dose of a TP therefore strengthens cytotype regulation significantly. The effect of the doubled dose is much greater than the doubled effect of a single maternally inherited TP. Strong regulation of P-element activity therefore involves synergism between the two elements in a TP homozygote.

Synergism between combinations of different telomeric P elements

Previous studies have not determined if two different TPs can interact to enhance cytotype regulation. To address this issue, we created combinations of TPs by performing reciprocal crosses between each of the TP strains and then tested these combinations for their ability to repress GD in the next generation (Figure 1). Females with combinations of NA and TP5 or NA and TP6 had a strong ability to repress GD in their offspring, no matter how the TPs were combined (≤12.3% GD). Thus, the NA element was able to interact genetically with either TP5 or TP6 to bring about strong regulation of P-element activity. By contrast, the two types of TP5/TP6 combinations had different abilities to repress GD—a strong ability (24.3% GD) when the TP5 element was maternally derived vs. a moderate ability (62.0% GD) when it was paternally derived. This difference indicates that a maternal effect can influence the genetic interaction between two telomeric P elements.

Presetting effects of telomeric P elements on cytotype regulation by paternally inherited telomeric P elements

Previous studies have shown that the strictly maternal (presetting) effects of TP5, TP6, and NA can enhance regulation by a paternally inherited TP (Marin et al. 2000; Niemi et al. 2004). To extend these studies, we performed an experiment to test if maternally transmitted factors from heterozygous TP w/+ , TP6 w/+ , or NA w/+ females could enhance the regulatory ability of a paternally inherited NA element linked to the wsp locus (Figure 2). In each generation, samples of females were test-crossed to Harwich y w males and their daughters were scored for GD. The results (Table 2) show that all the F1 TP w/+ heterozygotes enhanced the regulatory capacity of the paternally inherited NA element through a strictly maternal (i.e., presetting) effect. In the F2, the control NA w/FM7 het females, whose +/+ mothers did not carry a potentially presetting TP, had negligible ability to repress GD in their daughters (97.6% GD). By contrast, the NA w/FM7 +/+ females whose F1 mothers carried a potentially presetting TP were able to repress GD in their daughters. This repression was most pronounced when the presetting element was TP5 (52.5% GD), but it was also statistically significant when the presetting element was either TP6 or NA (88.0% GD and 92.1% GD, respectively). The other data in Table 2 document the regulatory properties of the various TPs in different situations. As expected, the TP homozygotes were moderate to strong repressors of GD in their daughters, the TP w/+ heterozygotes were weak to moderate repressors, and the TP/NA combinations were very strong repressors. From this experiment, we see that presetting by maternally transmitted factors can play a role in the emergence of strong cytotype regulation in females that carry two TPs.

Figure 2 Scheme to test for presetting of a paternally inherited TP by another TP. The presetting elements TP5, TP6, and NA originated in the females of the P generation; each of these elements was tightly linked to a null allele of the wsp locus. In the crosses involving TP5 and TP6, these females were homozygotes, whereas in the cross involving NA, they were NA w/FM7 heterozygotes. The target of presetting by these TPs was an NA element linked to the wsp allele. The wild-type flies that were used in the initial crosses came from the M strain Samar-kand. The different eye color
Table 2 Gonadal dysgenesis in the daughters of test crosses to detect the presetting effects of TPs on the telomeric element NA

| TP    | F1 TP w/+ Heterozygotes | F2 Synergism | F2 Presetting Effect |
|-------|--------------------------|--------------|----------------------|
|       | No. Vials | No. Flies | %GD ± SE | No. Vials | No. Flies | %GD ± SE | No. Vials | No. Flies | %GD ± SE |
| NA w6 | 25       | 360      | 4.0 ± 1.0 | 27       | 567      | 97.6 ± 0.8 |
| None (+) | 25         | 335      | 99.2 ± 0.8 | 28       | 841      | 52.5 ± 4.5 |
| TP5 w | 20       | 397      | 8.3 ± 1.6 | 28       | 712      | 3.6 ± 0.9 | 28       | 948      | 88.0 ± 2.0 |
| TP6 w | 24       | 367      | 57.6 ± 5.5 | 30       | 782      | 53.2 ± 1.7 | 30       | 844      | 92.1 ± 2.1 |

Gonadal dysgenesis was assessed in the daughters of test crosses between the various types of females obtained through the scheme in Figure 2 and Harwich y w males. In segregating crosses, different genotypes were scored, but because there were no differences between them, the results have been pooled.

- These F1 heterozygotes were obtained by crossing TP w/TP w homozygotes to + males from the M strain Samarkand, except in the case of NA w, where the cross was NA w/Fl males × F w males.
- Synergism between a TP and the NA element was assessed by testing TP w/NA w6 F2 females from crosses between TP w/+ F1, heterozygotes and NA w6 males (see Figure 2).
- The presetting effect of a TP on the NA element was assessed by testing +/NA w6 F2 females from crosses between TP w/+ F1 heterozygotes and NA w6 males (see Figure 2).
- Unweighted average percentage GD ± SE.
- The wild-type flies came from the M strain Samarkand, which is devoid of P elements.
- These data were obtained from tests with the +/NA w6 daughters of crosses between Samarkand (+) females and NA w6 males—that is, from the control cross in Figure 2.
- Females homozygous for the NA w chromosome produce many eggs that do not hatch, a form of sterility that is unrelated to hybrid dysgenesis. Consequently, this chromosome was maintained with the FM7 balancer in heterozygous condition, which may explain why the presetting effect of this NA element (see rightmost column) on a paternally inherited NA element is so weak.

Mutational disruption of synergism between two telomeric P elements

Previous studies have implicated the proteins encoded by the aub, piwi, and Su(var)205 genes as important factors in cytotype regulation (Ronsseray et al. 1996; Reiss et al. 2004; Haley et al. 2005; Josse et al. 2007; Simmons et al. 2007b, 2010). However, these studies have not addressed if mutational depletion of these proteins disrupts the synergism between two TPs. To investigate this issue, we assessed the regulatory abilities of TP5/NA females whose TP5 element came from a stock that was heterozygous for an aub, piwi, or Su(var)205 mutation; the tested females were also heterozygous for this mutation. As controls, we used stocks that were heterozygous for Gla, a mutation that has not been implicated in any aspect of cytotype regulation.

The end-points in this experiment were the GD frequencies among the daughters of the tested females. These frequencies could reflect the immediate effect of the mutation in the female’s genotype, or a cumulative (multi-generational) effect of the mutation in the stock from which the TP5 element and the mutation were derived. The different female genotypes were created in three sets of crosses. In cross 1, the TP5 element (linked to a null allele of w) and the mutation were maternally inherited, whereas in crosses 2 and 3, they were paternally inherited. The NA element (linked to the w6 allele) that could interact genetically with TP5 was inherited paternally in cross 1 and maternally in crosses 2 and 3; however, in cross 3, the NA element was transmitted from heterozygous rather than homozygous mothers— a condition that might diminish its regulatory ability. Thus, cross 3 provided an opportunity to assess the effects of the various mutations in TP5/NA females that might be more sensitive to these effects. The results of all the test crosses are summarized in Table 3.

The Gla control at the top of Table 3 shows that synergism between TP5 and NA in crosses 1 and 2 led to very strong repression of dysgenesis (<5% GD). In cross 3, this repression was not as strong (33.5% GD), indicating that, as hypothesized, the synergism between NA and TP5 is weakened when the NA element is inherited from heterozygous mothers. The Gla control in which TP5 was absent shows that by itself, a heterozygous NA element inherited from homozygous mothers leads to moderate repression (66.5% GD), but when inherited paternally or from heterozygous mothers, its repression ability is negligible (≥95.9% GD). The Gla control in which NA was absent shows that by itself, a heterozygous TP5 element inherited paternally or from heterozygous mothers has negligible repression ability (≥97.1% GD).

Among the mutations tested, Su(var)205 had the greatest impact on synergism between TP5 and NA. In all three crosses, this mutation profoundly disrupted the ability of the TP5/NA females to repress GD in their daughters (≥89.6% GD). This tilling effect is consistent with published data showing that Su(var)205 significantly impairs regulation by a single TP (Ronsseray et al. 1998; Marin et al. 2000; Haley et al. 2005; Belincio et al. 2009; Simmons et al. 2010). The aub and piwi mutations had less detrimental effects. Both mutant aub alleles moderately weakened cytotype regulation in the TP5/NA females from cross 1 (≥25.3% GD compared with the control value 0.5%)—that is, when the TP5 element and the aub mutation were inherited maternally, but they had much smaller effects in the females from cross 2 (≥13.3% GD compared with the control value 4.9%) or cross 3 (<43.0% GD compared with the control value 33.5%), where TP5 and the aub mutation were inherited paternally. These results indicate that the aub mutations impair synergism between two TPs through a maternal effect. The piwi mutations had little or no detrimental effects on the synergism between TP5 and NA in crosses 1 and 2; however, in cross 3, where the NA element came from heterozygous mothers, these mutations significantly impaired regulation by the TP5/NA combination (80% GD compared with the control value 33.5%). Thus, the piwi mutations impair regulation through a zygotic effect in TP5/NA females that already have a diminished capacity for regulation because they inherited their NA element from heterozygous mothers.

Synergism between telomeric and nontelomeric P elements

The telomeric elements TP5 and TP6 interact genetically with nontelomeric P elements to bring about very strong cytotype regulation (Simmons et al. 2007a, 2012). To see if regulation by the telomeric element NA could also be strengthened by genetic interactions with
nontelomeric P elements, we combined this element with the numerous nontelomeric P elements on the autosomes of the M′ strain Muller-5 Birmingham, here denoted simply as Birm. The procedure was to perform reciprocal crosses between the NA and Birm strains: NA females × Birm males (cross A), and NA males × Birm females (cross B). The F1 daughters of these crosses were then test-crossed to Harwich y w males to produce F2 females that were scored for GD according to whether they inherited the NA element, which was tightly linked to the w⁰ marker. For controls, we produced NA w⁰/+ F1 females by reciprocally crossing NA w⁰ flies to flies of the M strain Samarkand, and we produced Birm/+ F1 females by reciprocally crossing flies from the Samarkand and Birm strains. The control F1 females from these pairs of reciprocal crosses were then crossed to Harwich y w males to induce GD in the F2. The results from this experiment (Table 4) warrant several conclusions.

First, because 100% of the offspring of both types of control Birm/+ F1 females had GD, the Harwich y w strain is a powerful inducer of GD and the Birm P elements are unable to repress GD. Second, the NA element is able to repress GD, especially when the NA w²/+ F1 females inherited NA maternally—that is, through cross A. The daughters of these females had much less GD (47.6%) than those derived from cross B (95.6%). Third, repression of GD is enhanced when NA acts in combination with the Birm P elements. In cross A, when NA acted alone, 47.6% of the F2 females had GD, whereas when it acted together with the Birm P elements, only 2.1% of them had GD. In cross B, when NA acted alone, 95.6% of the F2 females had GD, whereas when it acted together with the Birm P elements, 41.6% of them had GD. The NA and Birm P elements therefore interact synergistically to regulate P activity, even when the NA/+; Birm/+ F1 females had inherited the NA element paternally. Fourth, NA-mediated regulation occurs in F2 females even when they do not inherit the NA element itself. In the tests with the NA w²/+; Birm/+ F1 females from cross A, GD was repressed almost as well as in the F2 daughters that did not carry NA (52.7% GD) as in those that did (46.0% GD). In the tests with the NA w²/+; Birm/+ F1 females from cross A, GD was repressed almost completely in both classes of daughters, and in the tests with the NA w²/+; Birm/+ F1 females from cross B, GD was repressed partially in both classes of F2 daughters (38.7% GD in those with NA and 47.3% GD in those without NA). Repression of GD is therefore mediated by a maternal effect established by NA, or by an interaction between NA and the Birm P elements, in the F2 females. The strength of this effect depends on whether an F1 female inherited NA maternally (moderate to strong repression) or paternally (weak to moderate repression), and on whether the Birm P elements were present (moderate to strong repression) or absent (weak to moderate repression) in the F2 female. Thus, the NA element has the same regulatory characteristics as the previously studied telomeric elements TPS and TP6 (Simmons et al. 2007a; Belinco et al. 2009).

Absence of presetting effects on and by nontelomeric P elements

Presetting by a maternal TP can enhance the regulatory ability of a paternally inherited TP. However, presetting by a maternal TP apparently does not enhance regulation by paternally inherited non-TPs (Belinco et al. 2009). We re-examined this issue by performing an experiment with the telomeric P elements TPS, TP6, and NA and the nontelomeric P elements of the M′ strain Muller-5 Birmingham, here denoted M5; Birm; this strain has previously been used to study interactions between TPs and non-TPs (Simmons et al. 2007a; Belinco et al. 2009). The scheme for the experiment is outlined and the results are summarized in Table 5. Several conclusions can be drawn from these results. First, GD was not repressed in the M controls (groups 1–3), nor when the Birmingham P elements acted alone (groups 4 and 5). Second, GD was repressed slightly or moderately by the TPs acting alone (groups 6 and 7, 11 and 12, and 16 and 17). NA had the greatest regulatory ability in these tests—64.2% GD in the F1 and 80.4% GD in the F2; TP6 had the next greatest—80.1% GD in the F1 and 96.6% GD in the F2; and TP5 had the least regulatory ability—96.2% GD in the F1 and 96.6% GD in the F2. NA was not repressed when these TPs were removed from the F2 genotype (groups 8, 13, and 18). Third, GD was repressed strongly by the TPs in combination with the nontelomeric Birmingham P elements (groups 9, 14, and 19), but it was not repressed at all by Birmingham P elements that had been exposed to the presetting effects of these TPs (groups 10, 15, and 20). Thus, collectively, the non-TPs in the M5; Birm strain are not susceptible to presetting by the TPS, TP6, or NA telomeric elements.
Another experiment determined if the regulatory ability of an individual non-TP could be influenced by presetting. This non-TP was a cloned version of TP5 contained within a hobo transgene inserted at map position 73.6 in the middle of chromosome 2R. The transgene, denoted H(hsp/TP5), is marked with a w^+ allele and has no intrinsic ability to repress GD; however, it and other insertions of H(hsp/TP5) can interact genetically with TP5, TP6, or NA to enhance cytotype regulation significantly (Simmons et al. 2012; Jessen et al. 2013). We determined if paternally inherited transgenic and telomeric TP5 elements were susceptible to the presetting effects of the telomeric elements TP5 and NA (Table 6).

A paternally inherited telomeric TP5 element had no ability to repress GD (group 0). However, this element did acquire weak, but statistically significant, repression ability when exposed to the presetting effects of either the TP5 (88.0% GD, group 4) or the NA (86.4% GD, group 10) telomeric elements. Both of these telomeric elements had some repression ability when they were maternally inherited; TP5 (group 1) repressed GD slightly (92.8%) and NA (group 7) repressed GD moderately (78.9%). However, when these elements were absent from the F1 genotype, GD was not repressed at all (groups 2 and 8). As expected, F2 females that carried two telomeric P elements repressed GD strongly—14.6% GD when the females carried two TP5 elements (group 3) and 5.1% GD when they carried NA and TP5 (group 9). F2 females that carried a maternally inherited telomeric element and a paternally inherited H(hsp/TP5) transgene also repressed GD strongly—6.9% GD when the maternally inherited element was TP5 (group 5) and 13.9% GD when it was NA (group 11). However, an H(hsp/TP5) transgene that had been exposed to the presetting effects of either TP5 (group 6) or NA (group 12) did not repress GD at all. Thus, like the diverse non-TPs in MS; Birm, the TP5 element within the H(hsp/TP5) is not susceptible to presetting by TP5 even though it can interact genetically with them to enhance cytotype regulation.

We also addressed the reciprocal issue—whether the H(hsp/TP5) transgene could preset a paternally inherited TP5 element. In this part of the analysis, w; H(hsp/TP5)D/+ females crossed to TP5 w males to obtain w/TP5 w; +/+ females (group 13) and w/TP5 w; H(hsp/TP5)D/+ females (group 14), which were then test-crossed to Harwich y w males. The females of group 13 could reveal if the repression ability of a paternally inherited telomeric TP5 element is enhanced by a strictly maternal (i.e., presetting) effect of the H(hsp/TP5)D transgene, and the females of group 14 could reveal if this ability is enhanced by the combined maternal and zygotic effects of the transgene. Nearly all (>98%) of the daughters from both sets of test crosses were dysgenic. Thus, the repression ability of a paternally inherited telomeric TP5 element is not enhanced by the maternal or zygotic effects of the H(hsp/TP5)D transgene.

### DISCUSSION

P elements inserted in the heterochromatic DNA at the XL telomere serve as anchors of cytotype regulation of P-element activity in the germ line. The effectiveness of this regulation can be assessed by measuring how well these TPs repress hybrid dysgenesis. Genetic analysis using the frequency of GD as the experimental end-point has shown that two TP—either structurally the same or different—establish very strong cytotype regulation in females, whereas a single maternally inherited TP represses GD modestly and a single paternally inherited TP does not repress GD at all. Cytotype regulation by two TP is therefore interactive rather than additive—that is, the regulatory effect of the two TP is much greater than the sum of their separate effects.

One event contributing to very strong cytotype regulation in females with two TP is the activation of the paternally inherited TP. Functionally active and inactive piRNA loci appear to produce the same steady-state levels of sense and antisense transcripts (De Vanssay et al. 2012). The activation of a paternally inherited TP therefore likely involves a posttranscriptional event that allows its transcripts—or transcripts that contain its sequence—to be processed into P-element piRNAs. P-element piRNAs synthesized in the mother’s germ line and transmitted through the egg cytoplasm may play a key role in this event, possibly by engaging with the transcripts of the TP to generate primary P-element piRNAs, or to initiate ping-pong cycling to generate secondary P-element piRNAs. In effect, the maternally transmitted P-element piRNAs preset the zygote to produce piRNAs from the paternally inherited transcripts of the TP. When a maternally transmitted TP is also present in the zygote, piRNA synthesis can be augmented by processing transcripts from this element as well, leading to enough P-element piRNAs to provide a strong defense against dysgenesis in future offspring. De Vanssay et al. (2012) have shown that two TPs generate approximately twice as many piRNAs as one maternally inherited TP. However, the regulatory effect of two TPs is much greater than twice the regulatory effect of a single maternally inherited TP. Thus, the strength of cytotype regulation is not simply proportional to piRNA abundance.

Presetting by maternally transmitted P-element piRNAs would be expected to play an important role in maintaining cytotype regulation in homozygous TP stocks. In each generation, these piRNAs would be needed to jumpstart the production of P-element piRNAs from the TPs in the genotype. Without a presetting effect, piRNA production would be sluggish and cytotype regulation would be impaired. Presetting also appears to influence the behavior of other piRNA loci. De Vanssay et al. (2012) found that an inactive piRNA locus in the middle of chromosome 2R could be activated by the presetting effect of an active “allele” of this locus, and that the activated locus remained...
active for many generations. However, the persistence of the active state may have depended on maternal transmission of the locus—and the piRNAs that it produced—over the course of the experiment; that is, the stable expression of piRNAs from this locus may have required the input of maternally transmitted piRNAs each generation.

The abundance and sequence complexity of maternally transmitted piRNAs are likely to influence the effectiveness of presetting. We found that the telomeric elements TP5, TP6, and NA could preset the activation of a paternally inherited NA element. Among these three presetting elements, NA had the weakest effect, possibly because it came from a heterozygous stock with a diminished ability to generate P-specific piRNAs. TP6 also had a weak presetting effect, but TP5 had a strong effect. Because TP5 shares more sequences with NA (1384 nucleotides) than TP6 does (1091 nucleotides), it would be expected to target a more complex array of piRNAs to the transcripts of NA and thereby enhance the prospects for these transcripts to be processed into piRNAs. Thus, the greater similarity between TP5 and NA may explain why TP5 is better able to preset the activation of NA.

The strong cytotype regulation that develops in females that carry two TPs was impaired by heterozygous mutations in the aub, piwi, and Su(var)205 genes. The aub mutations acted in the mothers of these females. Aub protein is located in the nuage, an indistinct region on the cytoplasmic side of the nuclear membrane where ping-pong cycling is thought to take place (Lim and Kai 2007; Kibanov et al. 2011; Nagao et al. 2011; Zhang et al. 2011; Anand and Kai 2012). In aub+/* females, the Aub protein may be depleted to such an extent that ping-pong cycling is impaired, leading to a smaller pool of piRNAs in the eggs of these females—too small, perhaps, to stimulate the production of P-element piRNAs from the TPs in their daughters. Another possibility is that Aub is involved in the transport of maternal piRNAs. Depletion of Aub may therefore curtail the delivery of piRNAs to the zygote.

The piwi mutations acted zygotically to impair cytotype regulation in females with two TPs. However, this effect was seen only when one of the TPs came from heterozygous TP/+ mothers, a condition that would be expected to diminish the abundance of maternally transmitted piRNAs in the eggs of these females. In their studies of the expression of piRNA genes, Wang and Elgin (2011) and Anand and Kai (2012) have found that piwi gene expression was diminished in the presence of P elements in the maternal genome. This effect may have been due to an increased abundance of the maternal piRNAs, which would block the production of Piwi protein by the paternal chromosome.

\[
\begin{array}{|c|c|c|c|c|c|c|} 
\hline
\text{Test Group} & \text{F1 Females}\text{a},\text{c} \times \text{F1 Males}\text{b} \rightarrow & \text{F2 Females}\text{c} & \text{No. Vials} & \text{No. Flies} & \%\text{GD} \pm \text{SE}\text{d} & \text{Issue Tested} \\
\hline
1 & w/+ & w/w & 25 & 456 & 100 & M strain control \\
2 & w/+ & w/w & 20 & 341 & 100 & M strain control \\
3 & M5; Birm & w/M5; +/-Birm & 25 & 500 & 99.8 \pm 0.2 & Effect of Birmingham P elements alone \\
4 & M5; Birm & w/M5; +/-Birm & 25 & 491 & 100 & Effect of Birmingham P elements alone \\
5 & TP5 w/+ & +/-w & 25 & 500 & 96.2 \pm 1.2 & Effect of TP5 alone in F1 \\
6 & TP5 w/+ & +/-w & 25 & 441 & 91.9 \pm 1.9 & Effect of TP5 alone in F2 \\
7 & TP5 w/+ & +/-w & 27 & 459 & 27.1 \pm 3.9 & Synergism between TP5 and Birmingham P elements \\
8 & TP6 w/+ & +/-w & 25 & 469 & 100 & Effect of removing TP5 in F2 \\
9 & TP6 w/+ & +/-w & 25 & 469 & 100 & Effect of removing TP5 in F2 \\
10 & NA w/+/+ & +/-w & 25 & 441 & 99.5 \pm 0.3 & Presetting effect of NA on Birmingham P elements \\
\hline
\end{array}
\]

*Four different types of F1 females that were heterozygous for a TP (or not, in the case of the controls) and a mutant w allele were crossed with two different types of F1 males to produce the various types of F2 females that were test-crossed to wild-type males from the M strain Samarkand. Samples of each of the four types of F1 females were also test-crossed with a heterozygous w w males. The daughters of all the test crosses were scored for GD without being sorted by genotype.

\text{a} F1 females were obtained by crossing females homozygous for a TP (or not, in the case of the controls) to wild-type males from the M strain Samarkand.

\text{b} F1 males came either from an M strain marked with a null allele of the TP5 (or not, in the case of the controls) to wild-type males from the M strain Samarkand. Samples of each of the four types of F1 females were also test-crossed with a heterozygous w w males. The daughters of all the test crosses were scored for GD without being sorted by genotype.

\text{c} First, the Aub protein may be depleted to such an extent that ping-pong cycling is impaired, leading to a smaller pool of piRNAs in the eggs of these females. In their studies of the expression of piRNA genes, Wang and Elgin (2011) and Anand and Kai (2012) have found that piwi gene expression was diminished in the presence of P elements in the maternal genome. This effect may have been due to an increased abundance of the maternal piRNAs, which would block the production of Piwi protein by the paternal chromosome.

\text{d} In these heterozygous genotypes, the maternally inherited components are written on the left side of the slashes.

\text{e} Unweighted average percentage GD ± SE.

The strong cytotype regulation that develops in females that carry two TPs was impaired by heterozygous mutations in the aub, piwi, and Su(var)205 genes. The aub mutations acted in the mothers of these females. Aub protein is located in the nuage, an indistinct region on the cytoplasmic side of the nuclear membrane where ping-pong cycling is thought to take place (Lim and Kai 2007; Kibanov et al. 2011; Nagao et al. 2011; Zhang et al. 2011; Anand and Kai 2012). In aub+/aub− females, the Aub protein may be depleted to such an extent that ping-pong cycling is impaired, leading to a smaller pool of piRNAs in the eggs of these females. In their studies of the expression of piRNA genes, Wang and Elgin (2011) and Anand and Kai (2012) have found that piwi gene expression was diminished in the presence of P elements in the maternal genome. This effect may have been due to an increased abundance of the maternal piRNAs, which would block the produc-
Table 6. Gonadal dysgenesis in the daughters of test crosses to detect the presetting effects of TPs with a transgenic P element

| Test Group | F$_1$ Females$^c$ × F$_1$ Males$^b$ → F$_2$ Females$^c$ | No. Vials | No. Flies | %GD ± SE$^d$ | Issue Tested |
|------------|-------------------------------------------------|-----------|-----------|---------------|--------------|
| 0          | y w/TP5 w                                       | 23        | 252       | 100           | Repression by paternally inherited telomeric TP5 element |
| 1          | TP5 w/y w                                       | 25        | 394       | 92.8 ± 1.7    | Repression by maternally inherited telomeric TP5 element |
| 2          | y w/y w                                         | 25        | 459       | 100           | Repression by cytoplasm from TP5 w/y w                   |
| 3          | TP5 w                                           | 21        | 236       | 14.6 ± 3.4    | Synergism between two telomeric TP5 elements             |
| 4          | y w/TP5 w$^w$                                   | 23        | 337       | 88.0 ± 2.5    | Presetting of one telomeric TP5 element by another        |
| 5          | y w, H(hsp/TP5)$^D$                             | 25        | 247       | 6.9 ± 1.7     | Synergism between telomeric TP5 and transgenic TP5 elements |
| 6          | y w/y w, H(hsp/TP5)$^D$/+                       | 25        | 283       | 100           | Presetting of transgenic TP5 element by telomeric TP5 element |
| 7          | NA w$^w$/y w                                    | 25        | 724       | 78.9 ± 2.5    | Repression by maternally inherited telomeric NA element |
| 8          | y w/y w                                         | 25        | 473       | 100           | Repression by cytoplasm from NA w$^w$/y w F$_1$ females |
| 9          | TP5 w                                           | 25        | 681       | 5.1 ± 1.4     | Synergism between telomeric NA and telomeric TP5 elements |
| 10         | y w/TP5 w                                       | 25        | 777       | 86.4 ± 1.8    | Presetting of telomeric TP5 element by telomeric NA element |
| 11         | y w, H(hsp/TP5)$^D$                             | 25        | 871       | 13.9 ± 4.0    | Synergism between telomeric NA and transgenic TP5 elements |
| 12         | y w/y w, H(hsp/TP5)$^D$/+                       | 25        | 698       | 100           | Presetting of transgenic TP5 element by telomeric NA element |
| 13         | w, H(hsp/TP5)$^D$/+                             | 19        | 173       | 98.8 ± 0.6    | Presetting of telomeric TP5 element by transgenic TP5 element |
| 14         | w/TP5 w, H(hsp/TP5)$^D$/+                       | 6         | 51        | 100           | Synergism between maternally inherited transgenic TP5 element and paternally inherited telomeric TP5 element |

Gonadal dysgenesis was assessed in the daughters of test crosses between F$_1$ females from group 0 and Harwich y w males and in the daughters of test crosses between F$_2$ females from groups 1–14 and Harwich y w males. Except where noted, data from the genotypes that segregated in the test crosses have been pooled.

- For group 0, F$_1$ females were obtained by crossing y w females from an M strain devoid of P elements to TP5 w males. For groups 1–6, F$_1$ females were obtained by crossing homozygous TP5 w females to y w males from this M strain. For groups 7–12, F$_1$ females were obtained by crossing homozygous NA w$^w$ females to y w males from this M strain. The y$^+$ allele present in the TP5 w and NA w$^w$ chromosomes is not shown. For groups 13 and 14, F$_1$ females were obtained by crossing homozygous w females from an M strain devoid of P elements to y w males.

- In these heterozygous genotypes, the maternally inherited components are written on the left side of the slashes.

- Unweighted average percentage GD ± SE.

- All the F$_1$ females that were scored carried the H(hsp/TP5)$^D$ transgene.

HP1 is found at many chromosomal locations, but mainly in the pericentric and telomeric heterochromatin (James et al. 1989). Mutational depletion of this protein might therefore disrupt the organization of heterochromatin. In addition, stocks that are heterozygous for a Su(var)205 mutation develop elongated telomeres (Savitsky et al. 2002). Together, these epigenetic and genetic changes could impair the production of piRNAs from the TPs by disrupting the transcription of the locus in which these elements are inserted, or by preventing maternally transmitted piRNAs from jumpstarting primary piRNA synthesis. Another possibility is that mutational depletion of HP1 interferes with the repressive modification of chromatin in and around P elements in the genomes of test-cross offspring, with the result that these elements are mobilized by the P transposase, ultimately causing dysgenesis.

Non-TPs interact synergistically with TPs to enhance cytotype regulation. The enhanced regulation is as strong as that created by synergism between two TPs and is transmitted to offspring independently of either the TP or the non-TP—that is, as a strictly maternal effect. The synergism between TPs and non-TPs is impaired by mutational depletion of HP1, Piwi, or Aub (Belinco et al. 2009) and is thought to reflect ping-pong amplification of P-element piRNAs (Simmons et al. 2012). It is much stronger when the TP is maternally inherited, presumably because the TP comes along with P-element piRNAs that jumpstart ping-pong cycling after fertilization. As examples, we found that three different TPs interacted strongly with non-TPs from the strain Muller-5 Birmingham, and that the two TPs tested (TP5 and NA) interacted strongly with the nontelomeric H(hsp/TP5)$^D$ transgene. Females carrying combinations of these maternally inherited TPs and paternally inherited non-TPs developed a strong ability to repress hybrid dysgenesis in their progeny. However, their sisters, which carried paternally derived non-TPs but did not carry a maternally derived TP, failed to develop this ability. This failure indicates that a zygotic effect of the TP is needed for the enhancement of cytotype regulation. The strictly maternal effect of the TP cannot...
elicit any regulatory ability from the Birmingham P elements or the \( H \) (hsp/TP)D transgene—that is, these non-TPs are not affected by pre-silencing. After fertilization, the P-element piRNAs associated with this maternal effect would be expected to initiate a ping-pong cycle fed by mRNAs transcribed from the paternally inherited non-TPs; however, without a TP to continue supplying primary piRNAs, this cycle is stymied.

We also found that a paternally inherited TP could not be preset by the strictly maternal effect of a non-TP. Thus, if RNAs from the non-TP are maternally transmitted, then they cannot elicit regulation from a paternally inherited TP. However, maternally inherited non-TPs can interact with a paternally inherited TP to bring about moderate to strong cytotype regulation (Table 4) (Simmons et al. 2007a, 2012). A plausible explanation is that as the paternally inherited TP begins to generate piRNAs—that is, as it is reactivated—these RNAs drive a ping-pong cycle fed by mRNAs from zygotic expression of the non-TPs. A population of secondary piRNAs then develops to regulate P-element activity.

Maternally transmitted P-element piRNAs play an important role in cytotype regulation. Without them, flies do not develop their full potential to repress hybrid dysgenesis. This arrested development implies that the small RNAs generated from the repetitive DNA of the TAS of XL—originally called repeat-associated small interfering (rasi) RNAs—are not so effective in triggering the production of piRNAs from paternally inherited TPs. However, these repeat-associated RNAs may be needed to maintain the heterochromatic state of the XL telomere. This state may minimize the chance for pairing between the repeated DNA within this telomere and similar DNA sequences at other telomeres. Such pairing could lead to chromosome nondisjunction during meiosis, or to inappropriate recombination. Thus, the repeat associated RNAs may primarily be involved in preventing chromosome entanglements that could lead to aneuploid gametes. However, loci that generate these RNAs clearly have acquired a secondary function: to regulate transposable elements. A transposon inserted into one of these loci is assimilated into a system that generates small RNAs with specificity to the transposon. As our genetic analysis of P elements in the TAS of XL shows, the entire transposon family then becomes regulated by the system for producing small RNAs. The prior existence of this and other epigenetic systems to maintain chromosomal integrity may be the reason that transposons are tolerated—and abundant—in eukaryotic genomes (Federror 2012).

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