Cytotype repression of hybrid dysgenesis in *D. melanogaster*: Limited synergism between a telomeric *P* element and individual non-telomeric *P* elements implies a failure in Ping-pong amplification of regulatory piRNAs.

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Transposable *P* elements are the cause of hybrid dysgenesis, a syndrome of germ-line abnormalities that includes frequent mutation, chromosome breakage, and sterility. This syndrome is repressed by the P cytotype, a maternally inherited state that is mediated by small RNAs generated by *P* elements inserted in the Telomere Associated Sequences (TAS) at the left end of the X chromosome (Brennecke *et al.*, 2008). Because these RNAs associate with the Piwi class of proteins, they are called Piwi-interacting, or "pi," RNAs. Cytotype regulation anchored in X-linked telomeric *P* elements is enhanced by *P* elements situated at non-telomeric loci, even though these other elements have no repression ability of their own (Simmons *et al.*, 2007, 2012). The enhanced regulation is thought to reflect a process that is fed by primary piRNAs from the telomeric *P* element and sense RNAs from the other *P* elements. With repetition, this process, called the ping-pong cycle (Aravin *et al.*, 2007; Gunawardane *et al.*, 2007), can amplify the population of *P*-specific piRNAs significantly.

Studies with the telomeric elements *TP5* and *TP6* have shown that all but the smallest non-telomeric *P* element can bring about synergistic repression of hybrid dysgenesis (Simmons *et al.*, 2012). These studies employed a set of *hobo* transgenes that contained different *P* elements (see Figure 1 in Simmons *et al.*, 2012): *SP* (naturally 0.5 kb long), *TP5* (1.8 kb long), *TP6* (1.9 kb long), *CP* (the complete, transposase-encoding *P* element, 2.9 kb long), and *P** (a frameshifted *P* coding sequence lacking the *P* promoter and all the *P* introns, 2.2 kb long). Each of these elements was terminally truncated and placed downstream of an *hsp70* promoter in a *hobo* transposon marked with the mini-*white* gene; the *SP*, *TP5*, *TP6*, and *CP* elements also possessed the natural *P*-element promoter. None of these *H*(*hsp*/*P*) transgenes have any intrinsic ability to repress GD, and none are located at a telomere (Jensen *et al.*, 2008; Simmons *et al.*, 2002, 2012).

We used the *H*(*hsp*/*P*) transgenes to determine what kinds of *P* elements could enhance cytotype regulation anchored in the telomeric *P* element *NA*-*P*(*1A*), which is deficient for the first 871 base pairs of the canonical *P*-element sequence (Marin *et al.*, 2000); hereafter, this telomeric *P* element is denoted simply as *NA*. Reciprocal crosses between an *NA* w^{sp} strain and each of several (*y*) *w*; *H*(*hsp*/*P*) strains were carried out; in cross A, the *NA* element was derived maternally and in cross B it was derived paternally. The *NA* w^{sp}/*w*; *H*(*hsp*/*P*)/+ F_1 daughters from these reciprocal crosses were then mated to Harwich *y w* males, which are powerful inducers of hybrid dysgenesis. The F_2 females from the F_1 matings were scored for dysgenic sterility (gonadal dysgenesis, or GD) according to standard procedures (Simmons *et al.*, 2012), but without regard to whether or not they carried *NA* or the *H*(*hsp*/*P*) transgene (Table 1).

In the absence of any transgene, the frequency of GD was 47.9% in cross A and 99.1% in cross B. Thus, by itself, the *NA* element was a strong repressor of GD, but only when it was inherited maternally by the F_1 females. Only one of the *NA*-*H*(*hsp*/*P*) transgene combinations—the one with *H*(*hsp*/*TP5*)*D*—produced significantly lower frequencies of GD than the controls; in cross A the frequency of GD was 2.7% and in cross B it was 84.2%. Thus, only the transgenic *TP5* element enhanced *NA*'s intrinsic ability to repress dysgenesis.

We performed two more experiments to test for synergism between *NA* and the transgenic *P* elements. They began with reciprocal crosses between flies from an *NA* y^+ *w*/*FM7*, sn^{x2} *B* strain and flies from different *y w*; *H*(*hsp*/*P*) transgenic strains. In cross A, *NA* y^+ *w*/*FM7* sn^{x2} *B* females were mated to *y w*; *H*(*hsp*/*P*) males and in cross B, *NA* y^+ *w* males were mated to *y w*; *H*(*hsp*/*P*) females. The *NA* y^+ *w*/*y w*; *H*(*hsp*/*P*)/+ F_1 females from both sets of crosses were mated to Harwich *y w* males to induce GD in their daughters, which were sorted by genotype and scored (Table 2); a diagram of a mating scheme similar to the one used in these experiments is presented in Figure 1 of Merriman and Simmons (2013).

By itself, *NA* was a very weak repressor of dysgenesis in the F_2 females from cross A in both experiments (94-97% GD), and it did not repress at all in the F_2 females from cross B (99.5-100% GD). The weakened repression ability in these controls may be due to the single dosage of *NA* in the females that were used to set up cross A. In the results reported in Table 1, where the control level of repression was stronger, cross A was initiated with females that had a double dose of *NA*.

In the first experiment we tested four different *H*(*hsp*/*P*) transgenes for interactions with *NA*. Only one of them, *H*(*hsp/TP5*)*D*, significantly enhanced repression in the F_2 females of cross A. The frequency of GD was roughly the same for all four classes of F_2 females from this cross (55-58%), and the F_2 flies that inherited neither *NA* nor *H*(*hsp*/*TP5*)*D* repressed GD as effectively as the flies that inherited both of these factors. Thus, the enhanced capacity for repression was transmitted independently of either the *TP* or the transgenic *P* element—that is, it operated as a strictly maternal effect. Only two of the transgenes, *H*(*hsp*/*CP*)*2* and *H*(*hsp*/*TP5*)*D*, were tested for synergism with *NA* in cross B; in these tests the frequency of GD was 98-100%. Thus, unlike the results obtained previously (Table 1), there was no evidence for synergism between *NA* and *H*(*hsp*/*TP5*)*D* in cross B, probably because the intrinsic level of repression by an *NA* element derived from a "single dose" *NA* stock is so low.

Table 1. Synergism between the telomeric *NA* element and various *H*(*hsp*/*P*) transgenes assessed in the daughters of *NA* w^{sp}/*w*; *H*(*hsp*/*P*)/+ F_1 females from reciprocal crosses between *NA* w^{sp} and (*y*) *w*; *H*(*hsp*/*P*) strains.

Transgene	Cross	No of vials	No of flies	%GD ± SE[a]
None	A	25	499	47.9 ± 4.8
H(*hsp*/*SP*)*A*	A	25	426	73.1 ± 4.2
H(*hsp*/*CP*)*2*	A	25	500	44.4 ± 2.0
H(*hsp*/*TP5*)*D*	A	21	413	2.7 ± 1.1
H(*hsp*/*TP6*)*C*	A	25	479	57.1 ± 4.6
H(*hsp*/*P**)*B*	A	25	470	36.9 ± 3.1
None	B	25	457	99.1 ± 0.4
H(*hsp*/*SP*)*A*	B	25	393	99.0 ± 0.6
H(*hsp*/*CP*)*2*	B	25	497	99.4 ± 0.3
H(*hsp*/*TP5*)*D*	B	25	493	84.2 ± 2.8
H(*hsp*/*TP6*)*C*	B	25	487	99.6 ± 0.3
H(*hsp*/*P**)*B*	B	25	498	98.8 ± 0.4

[a] Unweighted average percentage GD ± standard error. The standard error was computed from the empirical variance among replicate cultures.

In the second experiment we determined if other insertions of the *H*(*hsp*/*TP5*) transgene could interact synergistically with the telomeric element *NA*. In these tests, significant synergistic repression of GD was seen in the F_2 females from cross A, the strongest being with *H*(*hsp*/*TP5*)*B* (30% GD compared to 70-73% with insertions *A*, *C* and *D*), and in all cases, the synergism between *NA* and the *H*(*hsp*/*TP5*) transgenes involved a strictly maternal effect. Only the *H*(*hsp*/*TP5*)*B* insertion was tested for synergism with *NA* in cross B, and none was observed.

Ping-pong cycling has been invoked to explain the synergistic repression of hybrid dysgenesis by telomeric and transgenic *P* elements (Simmons *et al.*, 2012). For this cycling to occur, the telomeric and transgenic *P* elements must be at least partially homologous. Cytotype regulation anchored in *NA* was enhanced by the small transgenic element *TP5*, but not the slightly larger transgenic element *TP6*. This difference might be due to greater homology (293 nucleotides) between *NA* and *TP5* than between *NA* and *TP6*.

However, increased homology is not a guarantee that a transgenic *P* element will enhance cytotype regulation, because the transgenic elements *CP* and *P**, which are substantially more homologous to *NA* than *TP5* is, did not increase *NA*'s regulatory power. This failure suggests that the transcripts of these two elements are unable to feed a ping-pong cycle anchored in the *NA* element. However, *CP* and *P** do robustly enhance cytotype regulation anchored in the telomeric elements *TP5* and *TP6* (Merriman and Simmons, 2013; Simmons *et al.*, 2012). *CP* and *P** sense RNAs are, therefore, able to feed a ping-pong cycle initiated by primary piRNAs from either of these telomeric *P* elements.

Table 2. Synergism between the telomeric *NA* element and various *H*(*hsp*/*P*) transgenes assessed in the daughters of *NA* y^+ *w*/*y w*; *H*(*hsp*/*P*)/+ females from reciprocal crosses between *NA*-bearing and *H*(*hsp*/*P*)-bearing strains.

Transgene	Cross	No. of vials	Neither		Transgene only		*NA* only		Both		Pooled overall	
			No. of flies	%GD ± SE[a]	No. of flies	%GD ± SE[a]	No. of flies	%GD ± SE[a]	No. of flies	%GD ± SE[a]	No. of flies	%GD ± SE[a]
None	A	38	631	97.7 ± 1.0			612	96.7 ± 1.7			1243	97.2 ± 1.2
H(*hsp/SP*)*A*	A	35	288	96.7 ± 2.9	295	97.1 ± 2.9	326	96.3 ± 2.9	300	95.1 ± 3.0	1209	96.3 ± 2.9
H(*hsp/CP*)*2*	A	44	333	93.3 ± 1.6	352	92.1 ± 1.9	330	94.8 ± 1.6	328	91.1 ± 1.8	1343	92.7 ± 1.3
H(*hsp/TP5*)*D*	A	41	322	58.0 ± 5.2	312	55.5 ± 5.4	284	57.1 ± 5.2	349	56.9 ± 5.3	1267	57.7 ± 4.8
H(*hsp/TP6*)*C*	A	37	267	98.6 ± 0.7	298	96.9 ± 1.5	287	98.5 ± 0.8	274	97.8 ± 1.0	1126	97.8 ± 0.5
None	B	28	524	100 ± 0			511	100 ± 0			1035	100 ± 0
H(*hsp/CP*)*2*	B	26	293	100 ± 0	311	99.5 ± 0.4	290	100 ± 0	286	99.7 ± 0.3	1180	99.8 ± 0.1
H(*hsp/TP5*)*D*	B	26	296	98.2 ± 1.3	311	98.3 ± 0.7	307	98.3 ± 1.0	315	97.6 ± 0.9	1229	98.2 ± 0.5
None	A	17	193	93.9 ± 3.1			210	94.0 ± 3.2			403	94.2 ± 3.1
H(hsp/TP5)A	A	34	327	68.5 ± 5.8	300	71.9 ± 5.8	355	71.1 ± 5.7	358	70.2 ± 5.6	1340	70.9 ± 5.6
H(hsp/TP5)B	A	21	140	34.0 ± 7.5	144	30.1 ± 7.1	189	24.4 ± 5.7	169	31.8 ± 6.9	642	30.2 ± 6.2
H(hsp/TP5)C	A	30	219	71.5 ± 6.3	239	68.8 ± 6.4	205	71.1 ± 6.8	226	65.2 ± 6.5	889	69.6 ± 6.1
H(hsp/TP5)D	A	21	191	73.5 ± 5.9	170	76.7 ± 6.5	185	73.4 ± 6.7	188	67.4 ± 7.1	734	72.8 ± 5.9
None	B	17	298	99.7 ± 0.3			269	99.2 ± 0.6			567	99.5 ± 0.3
H(hsp/TP5)B	B	12	130	90.7 ± 3.9	118	97.1 ± 1.5	144	96.5 ± 1.6	106	97.9 ± 1.6	498	94.9 ± 1.6

In cross A, *NA* y^+ *w*/*FM7*, sn^{X2} *B* females were mated to *y w*; *H*(*hsp*/*P*) males, and in cross B, *NA* y^+ *w* males were mated to *y w*; *H*(*hsp*/*P*) females. The F_1 females resulting from these two sets of crosses were then mated to Harwich *y w* males to induce GD in their daughters, which segregated into four genotypic classes—carrying *NA* or not, and carrying the *H*(*hsp*/*P*) transgene or not. The data in the upper part of the table were collected from an experiment to survey different types of transgenes for interactions with the *NA* element; those in the lower part were collected from another experiment to test for synergism between *NA* and different insertions of the *H*(*hsp*/*TP5*) transgene.

[a] Unweighted average percentage GD ± standard error. The standard error was computed from the empirical variance among replicate cultures.

Why do *CP* and *P** enhance regulation anchored in *TP5* or *TP6*, but not in *NA*? One possibility is that with *NA* as the anchoring element, the longer RNAs from *CP* and *P** actually get in the way of ping-pong cycling. These sense RNAs might base pair with antisense transcripts from *NA* to form comparatively long double-stranded molecules that are diverted from the ping-pong cycle into an RNA interference pathway, where they generate siRNAs instead of piRNAs. Greater unbroken homology between telomeric and transgenic *P* RNAs may, therefore, prevent the production of enough piRNAs to repress hybrid dysgenesis strongly. The absence of synergism between the telomeric element *NA* and the transgenic elements *CP* and *P** implies that RNAs from these combinations of *P* elements interact in ways that keep them from entering the ping-pong cycle.

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References: Aravin, A.A., G.J. Hannon, and J. Brennecke 2007, Science 318: 761-764; Brennecke, J., C.D. Malone, A.A. Aravin, R. Sachidanandam, A. Stark, and G.J. Hannon 2008, Science 322: 1387-1392; Engels, W.R., 1979, Genet. Res. 33: 219-236; Gunawardane, L.S., K. Saito, K.M. Nishida, K. Miyoshi, Y. Kawamura, T. Nagami, H. Siomi, and M.C. Siomi 2007, Science 315: 1587-1590; Jensen, P.A., J.R. Stuart, M.P. Goodpaster, J.W. Goodman, and M.J.

Simmons 2008, Genetics 179: 1785-1793; Marin, L., M. Lehmann, D. Nouaud, H. Izaabel, D. Anxolabéhère, and S. Ronsseray 2000, Genetics 155: 1841-1854; Merriman, P.J., and M.J. Simmons 2013, Mol. Genet. Genomics 288: 535-547; Simmons, M.J., K.J. Haley, C.D. Grimes, J.D. Raymond, and J.B. Niemi 2002, Genetics 161: 195-204; Simmons, M.J., J.B. Niemi, D.-F. Ryzek, C. Lamour, J.W. Goodman, W. Kraszkiewicz, and R. Wolff 2007, Genetics 176: 1957-1966; Simmons, M.J., L.M. Ragatz, I.R. Sinclair, M.W. Thorp, J.T. Buschette, and C.D. Grimes 2012, Genet. Res. 94: 339-351.

Repression of hybrid dysgenesis in *D. melanogaster* males by the X-linked telomeric *P* element *NA-P(1A)*.

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Repression of hybrid dysgenesis is anchored in *P* elements that have inserted in the Telomere Associated Sequences (TAS) at the left end of the X chromosome. These telomeric *P* elements confer the P cytotype, a regulatory state that is mediated by small RNAs that interact with the Piwi class of proteins; the RNAs are, therefore, called Piwi-interacting, or "pi", RNAs. *NA-P*(*1A*) is a telomeric *P* element that produces *P*-specific piRNAs (Brennecke *et al.*, 2008). This element, hereafter denoted simply as *NA*, is inserted at the junction of the distal retrotransposon array and the TAS of chromosome XL (Marin *et al.*, 2000). The *NA* element is structurally incomplete, lacking the first 871 base pairs of the canonical *P*-element sequence, including the *P* promoter, the first *P* exon, the first *P* intron, and half of the second *P* exon; consequently, it cannot encode the transposase that catalyzes *P*-element activity or a truncated polypeptide that might interfere with this activity. The discovery that this element represses hybrid dysgenesis was a strong indication that cytotype regulation does not involve *P*-encoded repressor polypeptides.

Marin *et al.* (2000) demonstrated that *NA* represses hybrid dysgenesis in females. To determine if it also represses dysgenesis in males, we used a genetic test that detects excisions of the *P* elements inserted in sn^w, a weak mutant allele of the X-linked *singed* bristle locus (Engels, 1979). Two incomplete *P* elements are inserted in the 5' untranslated region of sn^w (Roiha *et al.*, 1988). Excision of the upstream element converts sn^w into sn^e, an allele with an extreme mutant phenotype. Excision of the downstream element converts sn^w into $sn^{(+)}$, a pseudo-wild allele. The extreme mutant and pseudo-wild phenotypes are easily distinguished from the weak mutant phenotype. We screened for *P*-element excisions from sn^w that occurred in the germ lines of males carrying sn^w and $H(w^+, \Delta 2\text{-}3)6$, a hobo transgene that produces the P transposase (Merriman and Simmons, 2013). These males were crossed to females with attached-X chromosomes so that sn^w or its sn^e or $sn^{(+)}$ derivatives would be inherited patroclinously. The sons of these crosses were then scored on days 14 and 17 for the three bristle phenotypes (weak, extreme, and pseudo-wild), and the frequency of the extreme and pseudo-wild sons was used to estimate the germ-line sn^w excision rate. A reduced rate indicates that *P* excisions have been repressed.

The $H(w^+, \Delta 2\text{-}3)6$ transgene contains a terminally truncated *P* element that lacks the last intron of the transposase gene—the one between exons 2 and 3 in a complete *P* element; hence its designation as $\Delta 2\text{-}3$. This transgene, inserted on chromosome 3, produces the P transposase in the soma as well as in the germ line. Genetic analyses have shown that like the widely used P transposase source $P(ry^+, \Delta 2\text{-}3)99B$ (Robertson *et al.*, 1988), $H(w^+, \Delta 2\text{-}3)6$ does not transmit