Research Notes

Can the *P* elements *TP5* and *TP6* affect repressor-sensitive alleles of the *singed* gene in *Drosophila melanogaster*?

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The incomplete *P* elements *TP5* and *TP6* were discovered as insertions in the telomere associated sequences (TAS) of chromosome XL in *D. melanogaster* (Stuart *et al.*, 2002). In their telomeric location, each of these elements brings about the P cytotype, a maternally inherited state that represses the movement of *P* elements in the germline. Cytotype regulation is mediated by small RNAs that interact with the Piwi family of proteins (Brennecke *et al.*, 2008, Jensen *et al.*, 2008). These Piwi-interacting (pi) RNAs are generated from many loci, including the one in the TAS of chromosome XL (Brennecke *et al.*, 2007). Before the discovery of telomeric *P* elements and piRNAs, cytotype regulation was thought to be mediated by repressor proteins encoded by the *P* elements themselves (Rio 1990). For example, complete *P* (*CP*) elements produce a 66 kd repressor polypeptide through alternate splicing of transcripts that encode an 87 kd transposase, and incomplete *P* elements first discovered in flies from Krasnodar, Russia (Black *et al.*, 1987)—hence, called *KP* elements—produce a 22 kd repressor polypeptide. These polypeptides act zygotically rather than maternally and, therefore, cannot explain cytotype regulation of the *P*-element family (Simmons *et al.*, 2002a, b). However, they may provide secondary controls on *P*-element activity in nature.

P-encoded repressor polypeptides affect the expression of some *P*-insertion mutations of the *singed* gene such as sn^{50e} and sn^{w} (Robertson and Engels 1989; Simmons *et al.*, 2004, Paterson *et al.*, 2007). The sn^{50e} allele exhibits repressor sensitivity in the soma, where the singed polypeptide is needed for normal bristle formation. In the absence of other *P* elements, sn^{50e} causes an extreme singed phenotype (very short, twisted bristles), but in the presence of repressor-encoding *P* elements, sn^{50e} flies have a moderate singed phenotype (longer, wavy bristles). The sn^{w} allele exhibits repressor sensitivity in the female germ line, where the singed polypeptide is needed for egg formation. In the absence of other *P* elements, homozygous sn^{w} females produce normal eggs, but in the presence of repressor-encoding *P* elements, sn^{w} homozygotes produce ill-formed eggs, which causes them to be less fertile or even sterile. This effect of repressor-encoding *P* elements, called "singed sterility," is also seen when sn^{w} is heterozygous with the radiation-induced null allele sn^{x2}.

In their telomeric locations, *TP5* and *TP6* have no discernable effect on these repressor-sensitive alleles (Simmons *et al.*, 2004). The lack of an effect could be due to the inability of these elements to encode repressor polypeptides, or to their inability to produce repressors because *P* insertions in the TAS generate piRNAs rather than mRNAs. To distinguish between these possibilities, we tested non-telomeric transgenes that contain either *TP5* or *TP6* for the ability to alter the phenotypes of sn^{50e} and sn^{w}. These transgenes, denoted in general as *H(hsp/P)* because they were constructed in a *hobo* (*H*)element vector and contain a fusion of the *P* element (with its promoter) to an ancillary promoter from the *D. melanogaster hsp70* gene, have been described (Simmons *et al.*, 2002a, b; Jensen *et al.*, 2008).

To see if the *H(hsp/P)* transgenes could suppress the sn^{50e} bristle phenotype, we examined hemizyous sn^{50e}; *H(hsp/P)*/+ males. In addition to transgenes containing *TP5* or *TP6*, we tested transgenes containing other types of *P* elements: *SP* (a 0.5 kb-long element, which is too short to encode a repressor polypeptide and could, therefore, serve as a negative control [Rasmussen *et al.*, 1993]), *KP* (which had never been tested in this assay), and *CP* (which is known to suppress sn^{50e} and could, therefore, serve as a positive control [Simmons *et al.*, 2004]). The bristle phenotype was observed in sons of the cross *w* sn^{50e} ♀♀ × w; *H(hsp/P)* ♂♂ which were reared at 25°C. All the crosses were also brooded to produce replicate cultures, which were subjected to a 1 hr treatment at 37°C on days 9, 10, and 11 after the cultures were established. Thus, it was possible to

score the phenotype in flies that had been subjected to a heat shock during the pupal stage, when the bristles develop, as well as in flies that had not (Table 1).

Table 1. Effect of *H(hsp/P)* transgenes on the singed bristle phenotype of males hemizygous for the repressor-sensitive allele sn^{50e}.

Transgene	Chromosome	Singed phenotype
H(*hsp*/*SP*)*A*	3	Extreme
H(*hsp*/*SP*)*B*	2	Extreme
H(*hsp*/*KP*)*3*	2	Extreme
H(*hsp*/*KP*)*7*[a]	2	Moderate
H(*hsp*/*KP*)*11*	2	Extreme/Moderate [b]
H(*hsp*/*KP*)*14*	2	Quasi-moderate
H(*hsp*/*TP5*)*A*	3	Extreme
H(*hsp*/*TP5*)*B*	2	Extreme
H(*hsp*/*TP5*)*C*	3	Extreme
H(*hsp*/*TP5*)*D*	2	Extreme
H(*hsp*/*TP6*)*A*	2	Extreme
H(*hsp*/*TP6*)*B*	3	Extreme
H(*hsp*/*TP6*)*C*	3	Extreme
H(*hsp*/*CP*)*2*	2	Moderate
H(*hsp*/*CP*)*3*	3	Moderate

[a] Two insertions of the transgene, separable by recombination, are located on chromosome 2.
[b] The moderate phenotype was seen only after heat shock treatments.

None of the *H(hsp/TP5)* or *H(hsp/TP6)* transgene insertions—7 insertions altogether—suppressed the sn^{50e} phenotype, even after heat shocks. The bristles of the flies that carried these transgenes were indistinguishable from those of the two *H(hsp/SP)* negative controls. By contrast, three of the four stocks with *H(hsp/KP)* insertions partially suppressed the mutant bristle phenotype, although one did so only after heat shock. In these cases, the bristles were identical to those of the two *H(hsp/CP)* positive controls. Thus, *KP*, but not *TP5* or *TP6*, produces a polypeptide that acts on the sn^{50e} *P* element to enhance expression of the *singed* gene in somatic cells.

To assess the effects of *P*-encoded polypeptides in germ-line cells, we examined the fertility of females heterozygous for sn^{w} and sn^{x2}. These sn^{w}/sn^{x2} females were obtained by crossing *FM7*, w^{81g} sn^{x2} *B*; *H(hsp/P)*/+ males to *w* sn^{w} females; w^{81g} is a null mutation of the *white* gene that arose spontaneously in the *FM7* balancer chromosome. Both types of daughters—those that inherited the *H(hsp/P)* transgene (recognized by their colored eyes) and those that did not (white eyes)—were allowed to mate with their brothers for 3-4 days, and then placed individually in 13 × 100 mm culture tubes supplied with a sugar-dried yeast medium (Simmons *et al.*, 1980); these tubes were then incubated at 25°C. Each female's fertility was quantified by counting the number of pupae present in the tube 9 days later.

The sn^{w}/sn^{x2} females that did not carry an *H(hsp/P)* transgene (right side of Table 2) were almost all fertile. The median number of offspring produced by these females ranged from 21 to 34, and the mean ranged from 21.4 to 34.8. The females that carried the *SP*, *TP5*, or *TP6* transgenes also largely conformed to this pattern. The highest frequency of sterility among these females was 0.13 for *H*(*hsp*/*TP6)B*; however, given the uncertainty in the data (binomial standard deviation 0.04), this frequency is not out of line with some of the frequencies observed among females that did not carry a transgene. Furthermore, the median and mean numbers of progeny produced by fertile females that carried the *SP*, *TP5*, or *TP6* transgenes were comparable to those of their transgene-free sisters. Thus, none of these transgenes appeared to cause singed sterility. In contrast, a considerable fraction of the females that carried the *KP* or *CP* transgenes were sterile, and among those that were fertile, the number of progeny was meager (median and mean numbers less than 10). The *H*(*hsp*/*KP*) insertions in two of the stocks (*3* and *7*) caused many females to be completely sterile (37 and 63 percent, respectively), and the insertions in all four of the *H*(*hsp*/*KP*) stocks caused significant reductions in fertility when compared to transgene-free flies. Both insertions of the *CP* transgene also caused some complete sterility, and they reduced the fertility of the non-sterile females significantly.

As a check on the specificity of this test for effects on the germ-line function of the sn^{w} allele, we also assessed the fertility of sn^{50e}/sn^{x2} females in the presence and absence of the *H(hsp/CP)* transgenes. Previous studies had indicated that the sn^{50e} allele is not associated with singed sterility—that is, sn^{50e}/sn^{x2} flies are fertile in the presence of P repressor polypeptides (Robertson and Engels 1989). Accordingly, we found that neither insertion of the *H(hsp/CP)* transgene caused sterility or reduced fertility in sn^{50e}/sn^{x2} females (last two rows in Table 2). In fact, the fertility of these females was greater than that of transgene-free sn^{w}/sn^{x2} females, suggesting that the sn^{w} allele has a fertility-reducing effect even in the absence of a *KP* or a *CP* transgene.

Table 2. Fertility of sn^{w}/sn^{x2} or sn^{50e}/sn^{x2} females in the presence and absence of *H(hsp/P)* transgenes.

	Transgene present					Transgene absent				
	No. of Females	Prop. sterile	No. of progeny per fertile female			No. of Females	Prop. Sterile	No. of progeny per fertile female		
			Median	Mean	SD[a]			Median	Mean	SD[a]
H(*hsp*/*SP*)*A*	53	0.02	22.5	22.8	9.1	44	0.00	27	26.3	9.1
H(*hsp*/*SP*)*B*	62	0.00	22.5	21.8	8.8	59	0.02	22.5	23.5	9.5
H(*hsp*/*KP*)*3*	62	0.37	2	3.2	3.5	57	0.02	27	26.3	9.4
H(*hsp*/*KP*)*7*	60	0.63	2.5	3.4	3.2	55	0.00	21	21.4	8.2
H(*hsp*/*KP*)*11*	51	0.04	8	9.6	6.9	58	0.09	26	25.1	8.7
H(*hsp*/*KP*)*14*	49	0.06	8	9.1	6.3	54	0.00	25	24.7	8.0
H(*hsp*/*TP5*)*A*	48	0.04	23	22.8	11.1	65	0.03	30	32.9	15.7
H(*hsp*/*TP5*)*B*	28	0.00	28.5	31.2	13.7	38	0.00	27.5	29.7	16.8
H(*hsp*/*TP5*)*C*	61	0.00	25	25.5	7.2	64	0.02	24	24.6	7.2
H(*hsp*/*TP5*)*D*	58	0.00	25.5	25.8	7.9	45	0.00	28	30.2	12.3
H(*hsp*/*TP6*)*A*	59	0.02	26	27.1	10.6	57	0.00	29	31.2	11.5
H(*hsp*/*TP6*)*B*	70	0.13	24	23.3	8.7	66	0.06	27	26.1	8.3
H(*hsp*/*TP6*)*C*	34	0.03	31	29.9	10.7	32	0.00	27.5	26.8	8.0
H(*hsp*/*CP*)*2*	84	0.11	5	6.8	5.8	57	0.02	34	34.8	13.1
H(*hsp*/*CP*)*3*	31	0.19	7	7.4	5.1	24	0.08	24	26.3	11.3
H(*hsp*/*CP*)*2*	36[b]	0.00	38.5	40.1	7.8	24[b]	0.00	44	45.1	6.3
H(*hsp*/*CP*)*3*	38[b]	0.00	39.5	37.0	10.7	30[b]	0.00	40	41.4	8.6

[a] Standard deviation
[b] These females were sn^{50e}/sn^{x2}; all others in the Table were sn^{w}/sn^{x2}.

Although the *KP* and *CP* transgenes significantly impaired the germ-line function of the sn^{w} allele, neither they nor any of the other transgenes affected its somatic function. In the presence of any of the *H(hsp/P)* transgenes, sn^{w} males have moderately twisted bristles that are somewhat shorter than wild type. Females heterozygous for sn^{w} and sn^{x2} also have moderately twisted bristles, regardless of the presence of an *H(hsp/P)* transgene. Thus, at the phenotypic level, the repressor sensitivity of the sn^{w} allele is limited to the female germ line. Furthermore, only *P* elements that encode known repressor polypeptides—*KP* and *CP*—bring out this sensitivity; the *TP5* and *TP6* elements, either in their native telomeric positions (Simmons *et al.*, 2004) or in transgenes designed to express them, do not.

Both *KP* elements and *CP* elements encode polypeptides that repress some aspects of hybrid dysgenesis, a syndrome of germ-line abnormalities caused by *P* activity, and both of these elements induce changes in the phenotypes of sn^{50e} and sn^{w}. This phenotype-changing ability is thought to involve the KP and 66 kD repressors binding to the *P* elements inserted in these mutant alleles. In one case (sn^{50e}), the phenotype is ameliorated, whereas in the other (sn^{w}), it is worsened. Although the bound repressors presumably influence transcription of the mutant *singed* gene—for better or for worse—the specific reasons for these opposite effects are unknown.

Both the KP and 66 kD repressors possess a domain that recognizes and binds to *P* elements at three different sites: the transposase-binding sites near the 31-bp terminal inverted repeats, the 11-bp internal inverted repeats, and—at high protein concentrations—the terminal inverted repeats (Lee *et al.*, 1996, 1998). It is plausible that when either type of repressor interacts with any of these sites, it affects transcription from the *P* promoter. Indeed, both types of repressors have been shown to silence the expression of *P-lacZ* transgenes *in vivo* (Lemaitre *et al.*, 1993; Lemaitre and Coen 1991). Repressor binding to complete *P* elements has been hypothesized to limit the production of the P transposase, and ultimately to reduce the amount of *P*-element movement. However, *P*-element movement might also be reduced if repressor binding

simply prevents the transposase from attacking potentially mobile *P* elements. The domain for *P*-element binding is located within the first 88 amino acids of the repressor polypeptide sequence (Lee *et al.*, 1998). Artificially engineered polypeptides with these amino acids are able to repress *P*-element transposition *in vitro*, although not as well as longer polypeptides. Thus, other regions in the KP and 66 kD polypeptides contribute significantly to repressor function.

Both the *TP5* and *TP6* elements could encode polypeptides longer than 88 amino acids, and transgenes designed to express the TP5 polypeptide, but not those designed to express the TP6 polypeptide, have a modest ability to repress transposase-induced sn^{w} mutability (Jensen *et al.*, 2008). However, this repression is not nearly so strong as that seen with the *H*(*hsp*/*KP*) transgenes, and it is very much weaker than that of the native *TP5* element situated in the TAS of the XL telomere. The TP5 polypeptide, which is 113 amino acids long, may, therefore, be a bona fide repressor, but it is not as effective as the KP repressor. Furthermore, unlike the KP and 66 kD repressors, the TP5 polypeptide has no ability to alter the phenotypes of sn^{50e} and sn^{w}. Repressors that alter mutant singed phenotypes may do so because they possess downstream amino acid motifs that augment their ability to bind to *P* elements. For instance, a leucine zipper in the KP polypeptide has been implicated in its ability to form dimers, and dimerization may enhance the ability of KP repressors to bind to *P*-element DNA. Andrews and Gloor (1995) demonstrated that this leucine zipper is important for repression of hybrid dysgenesis. One other fact argues that *TP5* and *TP6* do not produce polypeptides with significant repressor function: Unlike *KP*, neither of these elements is widespread in natural populations (Stuart *et al.*, 2002). If *TP5* and *TP6* encoded effective repressor polypeptides, we would expect natural selection to have brought them to noticeable frequencies in many places in the world.

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Evidence for action of the KP repressor polypeptide in the germ line of female *Drosophila melanogaster* carrying piRNA-generating telomeric *P* elements.

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The transposable *P* elements of *D. melanogaster* are responsible for a syndrome of germ-line abnormalities that includes sterility and high frequencies of mutation and chromosome breakage (Engels, 1989). This syndrome, called hybrid dysgenesis, occurs in the offspring of crosses between paternally contributing (P) and maternally contributing (M) strains. Thus, P male × M female produces dysgenic offspring, but P female × M male usually does not. Hybrid dysgenesis is repressed by small *P*-derived RNAs,