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Cytotype Regulation by Telomeric *P* Elements in *Drosophila melanogaster*: Evidence for Involvement of an RNA Interference Gene

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ABSTRACT

P elements inserted at the left telomere of the *X* chromosome evoke the P cytotype, a maternally inherited condition that regulates the *P*-element family in the Drosophila germline. This regulation is completely disrupted in stocks heterozygous for mutations in *aubergine*, a gene whose protein product is involved in RNA interference. However, cytotype is not disrupted in stocks heterozygous for mutations in two other RNAi genes, *piwi* and *homeless* (*spindle-E*), or in a stock heterozygous for a mutation in the chromatin protein gene *Enhancer of zeste*. *aubergine* mutations exert their effects in the female germline, where the P cytotype is normally established and through which it is maintained. These effects are transmitted maternally to offspring of both sexes independently of the mutations themselves. Lines derived from mutant *aubergine* stocks reestablish the P cytotype quickly, unlike lines derived from stocks heterozygous for a mutation in *Suppressor of variegation 205*, the gene that encodes the telomere-capping protein HP1. Cytotype regulation by telomeric *P* elements may be tied to a system that uses RNAi to regulate the activities of telomeric retrotransposons in Drosophila.

SINCE its discovery by Fire *et al.* (1998), RNA interference (RNAi) has been found to play an important role in the expression of genes in diverse organisms. It has also been implicated in the control of transposable genetic elements. In *Drosophila melanogaster*, for example, RNAi appears to regulate the levels of RNAs derived from several kinds of retrotransposons, including elements with long terminal repeats and elements without these repeats (Vagin *et al.* 2006), and in *D. virilis*, it has been implicated in the regulation of the retroelement *Penelope* (Blumenstiel and Hartl 2005). In this article, we test the hypothesis that the *P* element, an important cut-and-paste transposon in the *D. melanogaster* genome, is regulated by RNAi. Our approach is genetic. Mutations in genes whose products are involved in RNAi are tested for impairment of *P*-element regulation.

Our study focuses on three RNAi genes: *aubergine* (*aub*), *piwi*, and *homeless* (*hls*, also known as *spindle-E*). The genes *aub* and *piwi* encode Argonaute-type proteins that are integral parts of an RNAi pathway in Drosophila. Evidence suggests that they bind small interfering RNAs and guide them to target RNAs, which may then be destroyed (Vagin *et al.* 2006). The *hls* gene encodes a putative helicase that also appears to play an important role in RNAi (Kennerdell *et al.* 2002). Mutations in all three genes have been shown to affect the levels of RNA produced by several different retrotransposons, including *I*, *gypsy*, and *HeT-A* (Vagin *et al.* 2006), and mutations in *aub* and *hls* have been shown to enhance transposition of the retrotransposon *TART*, which is a component of Drosophila telomeres (Savitsky *et al.* 2006). Mutations in *aub*, *piwi*, and *hls* also seem to alter the distribution of certain proteins on chromosomes (Pal-Bhadra *et al.* 2004), which suggests that their products influence chromatin organization as well as mRNA levels. More to the point, Reiss *et al.* (2004) have reported that mutations in *aub* disrupt an aspect of *P*-element regulation in the germline.

Our study includes one other gene, *Enhancer of zeste* [*E(z)*], whose product is a chromosomal protein involved in chromatin organization and the control of gene expression. This gene was implicated in *P*-element regulation by Roche and Rio (1998), although reservations about some of their results have been expressed (Rio 1999).

P-element regulation is complex, and disentangling the mechanisms that are involved in it has been difficult. In the soma, *P* activity is regulated by a mechanism that prevents the removal of the last of the three introns in primary *P* transcripts (Rio 1990). In the germline, all three introns are removed to create an mRNA that encodes an 87-kDa polypeptide, the P transposase, which catalyzes the excision and insertion of *P* elements.

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Because this transposase is produced only in the germline, *P*-element activity is restricted to that tissue (Laski *et al.* 1986; Rio *et al.* 1986).

P activity is further regulated by a state called the P cytotype (Engels 1989). This state is characteristic of most strains that have *P* elements in their genomes. Because the P cytotype is repressive, the *P* elements in these strains are quiescent. However, they can be mobilized if males from a P strain are crossed to females from a strain that lacks *P* elements. Such females pass on to their offspring a condition called the M cytotype, which permits *P*-element movement. When *P* elements are brought into the M cytotype by this type of cross, they cause a syndrome of germline abnormalities called hybrid dysgenesis. This syndrome is characterized by sterility, chromosome breakage, and high mutation rates (Kidwell *et al.* 1977). Hybrid dysgenesis does not occur, or occurs infrequently, in offspring from the reciprocal cross, P female × M male, because P females transmit the repressive P cytotype through their eggs. The conspicuous difference between the genetically identical offspring of these reciprocal crosses was the primary evidence that cytotype regulation involves a maternal component. Early studies indicated that the P cytotype is determined by the *P* elements themselves (Engels 1979a; Kidwell 1981). More recent analyses have shown that it can be established and maintained by *P* elements in special genomic locations (Ronsseray *et al.* 1991; Marin *et al.* 2000; Niemi *et al.* 2004; Simmons *et al.* 2004).

For many years cytotype regulation has been thought to involve *P*-element-encoded polypeptides, for instance, a 66-kDa polypeptide encoded by complete *P* elements when the last *P* intron is retained in the mRNA (Rio 1990). Experiments have shown that this polypeptide does function as a repressor of *P* activity (Misra and Rio 1990) and that polypeptides encoded by some incomplete *P* elements are also repressors (Black *et al.* 1987; Andrews and Gloor 1995). However, because these types of polypeptides do not appear to be produced in some strains that clearly do have the P cytotype, the hypothesis of cytotype regulation by P polypeptides has been questioned (Stuart *et al.* 2002; Simmons *et al.* 2004; P. Jensen, J. Stuart, M. Goodpaster, K. Newman, J. Goodman and M. Simmons, unpublished results).

Key insights into the nature of cytotype regulation have been obtained by studying strains that have *P* elements inserted into the *t*elomere-*a*ssociated *s*equences (TASs) at the left end of the *X* chromosome. Extensive analyses by Stéphane Ronsseray, Dominique Anxolabéhère, and colleagues have shown that these elements can confer the P cytotype on their carriers (Ronsseray *et al.* 1991, 1996, 1998; Marin *et al.* 2000). Stuart *et al.* (2002) added to this evidence by analyzing the regulatory abilities of two incomplete *P* elements, *TP5* and *TP6*, inserted in the TAS at the left end of the *X* chromosome. Further study of these elements has shown that they regulate *P* activity in the germline but not in the soma, that their regulatory abilities are established and maintained in the female germline, that these abilities are passed on to offspring of either sex, and that, in at least some cases, they are transmitted to offspring independently of the telomeric *P* elements themselves (Niemi *et al.* 2004; Simmons *et al.* 2004, 2007, accompanying article in this issue). However, neither *TP5* nor *TP6* appears to encode a polypeptide with any significant repressor function (Stuart *et al.* 2002; P. Jensen, J. Stuart, M. Goodpaster, K. Newman, J. Goodman and M. Simmons, unpublished results). Thus, their ability to repress hybrid dysgenesis has been hypothesized to involve an RNA, which raises the possibility that cytotype regulation of *P* elements is mediated by an RNA interference mechanism.

To explore this idea, we incorporated RNAi mutations into stocks carrying *TP5* or *TP6* and then tested these stocks for repression of *P* activity. Because the RNAi mutations are either homozygous lethal or sterile, we were able to test only for heterozygous effects. Despite this limitation, however, we have obtained evidence that at least one of three RNAi genes—*aubergine*—is required for cytotype regulation of the *P*-element family.

MATERIALS AND METHODS

Drosophila stocks and husbandry: Information on the genetic markers and special chromosomes in the stocks used in this analysis is available at the FlyBase website (http://flybase.bio.indiana.edu/), in Lindsley and Zimm (1992), or in other references cited in the text. The P cytotype strains that were analyzed carried an *X* chromosome with an incomplete *P* element (either *TP5* or *TP6*) inserted in one of the repeats within the TASs at the left end of the *X* chromosome; the *TP5* element is 1.8 kb long and the *TP6* element is 1.9 kb long. Although these elements are inserted at the same position in the TAS repeat, strains carrying the *TP5* element consistently repress *P*-element excision more strongly than strains carrying the *TP6* element (Stuart *et al.* 2002; Simmons *et al.* 2004). The *X* chromosomes carrying *TP5* or *TP6* were marked with the *w* mutation, which is tightly linked to the left telomere of the *X* and therefore serves as a visible marker for the telomeric *P* element (Stuart *et al.* 2002). The *E(z)*, *aub*, *hls*, and *piwi* mutations, along with appropriate recombination-suppressing balancer chromosomes, were crossed into these P cytotype strains and into control M strains and then maintained as balanced stocks. All cultures were reared on a cornmeal–molasses–dried yeast medium. Stock cultures were maintained at 18°–21° and experimental cultures were maintained at 25°.

Assay for *P*-element excision: The basic M and P cytotype strains, and all the mutant strains derived from them, carried a hypermutable allele of the *X*-linked *singed* gene (sn^w, *singed weak*). In hemizygous males, this allele causes a moderate malformation of the bristles. In homozygous females, it has little or no phenotypic effect; however, when sn^w is heterozygous with an extreme allele of the *singed* gene, such as sn^3 or sn^{x2}, the bristle phenotype is similar to that of hemizygous sn^w males.

The sn^w allele is due to the insertion of two incomplete *P* elements in the 5′ untranslated portion of the *singed* gene. In the presence of the P transposase, either of these *P* elements

can be excised. However, because these excisions occur in the germline, their phenotypic effects are not visible until the next generation. If the upstream *P* element is excised, the resulting flies have extremely malformed bristles (sn^e); if the downstream *P* element is excised, they have wild-type bristles (sn^+). The frequency of these altered phenotypes therefore indicates the rate of *P*-element excision in the parental germline. For males, this quantity was assessed by crossing individual sn^w males that carried a source of the P transposase to three *C(1)DX* females. Because these females have attached-*X* chromosomes, their sons inherit sn^w or its derivatives patroclinously. Thus, the combined frequency of the wild type and extreme singed sons among all the sons counted was used to estimate the *P*-element excision rate. For females, the excision rate was assessed by crossing individual sn^w/sn^+ females that carried a source of the P transposase to three sn^3 males. Because the tested females carried a preexisting sn^+ allele, only their extreme singed progeny provided information about *P*-element excisions occurring in the germline. Consequently, the *P* excision rate was estimated by calculating the frequency of the sn^e flies among all the sn^w and sn^e flies of both sexes.

In addition to the telomeric *P* elements *TP5* and *TP6*, the only other *P* element present in the stocks that were analyzed for excision events was a 0.6-kb-long element tightly linked to the sn^w allele. This element is situated in a different cytological position than *singed* (band 7D5-6 *vs.* band 7D1-2 for *singed*) and is referred to as the "unsinged" element (Roiha *et al.* 1988).

All the experiments to measure the frequency of *P*-element excisions were carried out with replicate cultures, and the offspring in these cultures were scored on days 14 and 17 after the cultures were established. All the data from different groups within an experiment were obtained within a 1- or 2-week period. The average excision frequency for each experimental group was calculated by treating all replicates equally—that is, with the unweighted average—and the associated variance was calculated empirically among the replicates. This procedure, which encompasses secular variation, sampling variation, and variation due to *P*-element excisions in premeiotic cells, is considered a conservative approach to the analysis of mutation rate data (Engels 1979b). Statistical differences between groups within experiments were evaluated by *t*- or *z*-tests using standard errors of the unweighted sample means.

RESULTS

Tests with the $E(z)^{28}$ mutation: Roche and Rio (1998) found that, in heterozygous condition, several alleles of the *E(z)* locus impaired the P cytotype conferred by *P* elements inserted in the *X*-linked TAS. However, the telomeric *P* insertions in their study were complete elements capable of producing the P transposase. Rio (1999) subsequently reported that these elements had been lost in some of the stocks used in their published experiments, thereby calling into question the evidence that *E(z)* mutations impair cytotype regulation. We chose one allele of the *E(z)* locus, $E(z)^{28}$, which Roche and Rio (1998) had found to impair the P cytotype strongly, to test for an effect on repression of *P*-element excisions from the sn^w allele in stocks that had incomplete (and therefore genetically stable) *P* elements inserted in the *X*-linked TAS.

TABLE 1

Effect of $E(z)^{28}$ on cytotype-mediated repression of *P* excisions from sn^w in the male germline

TP	Genotype[a]	No. of vials	No. of flies	Excision rate ± SE[b]
None	+/+	50	1460	0.536 ± 0.013
None	$E(z)^{28}/+$	48	1077	0.473 ± 0.022
TP5	+/+	49	1270	0
TP5	$E(z)^{28}/+$	48	1185	0.003 ± 0.002
TP6	+/+	49	1276	0.055 ± 0.011
TP6	$E(z)^{28}/+$	49	1285	0.058 ± 0.010

[a] Genotypes at the *E(z)* locus of males that were tested for *P* excisions from sn^w. These males were also heterozygous for the *H(hsp/CP)2* transgene, which encodes the P transposase. Thus, the genotype of the tested males was (*TP*) sn^w; *H(hsp/CP)2/+*; $E(z)^{28}$ or $E(z)^+/+$.

[b] Average unweighted excision rate $[(sn^+ + sn^e)/(sn^w + sn^+ + sn^e)]$ ± standard error.

These tests were initiated by crossing sn^w; $E(z)^{28}/TM3$, *Sb Ser* females to males homozygous for *H(hsp/CP)2*, a transgene inserted on chromosome *2* that encodes the P transposase (Simmons *et al.* 2002). In these crosses, one group of females was homozygous for the *TP5* element and another group was homozygous for the *TP6* element. Previous studies have indicated that both of these telomeric *P* elements bring about the P cytotype (Stuart *et al.* 2002). A third group of females carried neither *TP5* nor *TP6*. The sn^w; *H(hsp/CP)2/+*; $E(z)^{28}/+$ sons from these three types of females were then crossed to females with attached-*X* chromosomes and their progeny were scored to assess the frequency of *P*-element excisions from sn^w that had occurred in the paternal germline. Control tests were carried out with sn^w; *H(hsp/CP)2/+* males derived from stocks that did not carry the $E(z)^{28}$ mutation.

The results of all these tests are shown in Table 1. Flies that did not carry a telomeric *P* element had *P* excision rates of 0.536 [in the absence of the $E(z)^{28}$ mutation] and 0.473 (in the presence of this mutation). The similarity of these numbers indicates that the $E(z)^{28}$ mutation did not affect the frequency of *P*-element excision *per se*. In flies that carried *TP5*, the respective excision rates were 0 and 0.003, and in flies that carried *TP6*, they were 0.055 and 0.058. These data indicate that both *TP5* and *TP6* strongly repressed *P* excisions from sn^w in the presence of $E(z)^{28}$ as well as in its absence. Thus, the $E(z)^{28}$ mutation does not impair cytotype-mediated repression of *P*-element excision.

Preliminary tests with *aub*, *hls*, and *piwi* mutations: A similar procedure was followed to ascertain if mutations in three RNAi genes—*aub*, *hls*, and *piwi*—had an effect on cytotype-mediated repression of *P* excisions from sn^w. *TP5* sn^w or *TP6* sn^w females that carried one of these mutations over a balancer chromosome were mated to *H(hsp/CP)2* males and their *TP* sn^w sons, which were

TABLE 2

Effects of mutations in the *aubergine*, *homeless*, and *piwi* genes on cytotype-mediated repression of *P* excisions from sn^w in the male germline

TP	Genotype[a]	No. of vials	No. of flies	Excision rate ± SE[b]
None	+/+	27	1235	0.469 ± 0.019
TP5	+/+	28	1312	0.087 ± 0.019
TP5	$aub^{\Delta P\text{-}3a}$/+	28	899	0.559 ± 0.024
TP5	aub^{QC42}/+	26	1086	0.393 ± 0.028
TP5	hls^{D58}/+	30	1147	0.102 ± 0.028
TP5	$hls^{\Delta 125}$/+	29	1164	0.089 ± 0.020
TP5	hls^{E616}/+	29	1230	0.017 ± 0.009
TP5	$piwi^1$/+	29	1070	0.057 ± 0.021
TP5	$piwi^2$/+	29	1087	0.026 ± 0.008
TP6	+/+	28	1526	0.240 ± 0.029
TP6	$aub^{\Delta P\text{-}3a}$/+	30	1617	0.499 ± 0.028
TP6	aub^{QC42}/+	29	1417	0.505 ± 0.027
TP6	hls^{D58}/+	30	1530	0.116 ± 0.023
TP6	$hls^{\Delta 125}$/+	30	1671	0.063 ± 0.017
TP6	hls^{E616}/+	29	1513	0.039 ± 0.012
TP6	$piwi^1$/+	25	1370	0.183 ± 0.026
TP6	$piwi^2$/+	29	1726	0.178 ± 0.025

[a] Genotypes of the *aubergine, homeless*, or *piwi* locus in the males that were tested for *P* excisions from sn^w. These males were also heterozygous for the *H(hsp/CP)2* transgene, which encodes the P transposase. Thus, the genotype of the tested males was (*TP*) sn^w; *aub* or *piwi*/*H(hsp/CP)2*; +/+ or (*TP*) sn^w; +/*H(hsp/CP)2*; *hls*/+. The *aub* and *piwi* mutations were maintained in stocks with the *Cy Roi* [= $In(2L)Cy^Lt^R$ + *In(2R)Cy, Cy Roi cn sp bw*] balancer chromosome; the *hls* mutations were maintained in stocks with the *TM6, Tb e* balancer chromosome.

[b] Average unweighted excision rate $[(sn^+ + sn^e)/(sn^w + sn^+ + sn^e)]$ ± standard error.

heterozygous for one of the mutations and the *H(hsp/CP)2* transgene, were tested for *P* excisions by crossing them to attached-*X* females. As controls, we tested sn^w; *H(hsp/CP)2*/+ and *TP* sn^w; *H(hsp/CP)2*/+ males that did not carry any of the mutations. The results of all these tests are shown in Table 2.

In the absence of either telomeric *P* element, the control *P*-excision frequency was 0.463. With *TP5* present, it was 0.087, and with *TP6* present, it was 0.240. Even though the latter numbers are greater than the corresponding excision frequencies in Table 1, they are still significantly <0.469. Thus, both *TP5* and *TP6* repressed *P* excisions from sn^w. Previous studies have indicated that *TP5* is a stronger repressor of *P* excision than *TP6* (Stuart *et al.* 2002; Simmons *et al.* 2004).

Among the *aub, hls*, and *piwi* mutations tested, only the *aub* alleles impaired *TP5*- and *TP6*-mediated repression of *P* excision. The excision frequencies for the flies that carried these alleles were similar to the frequency for the flies that did not carry either telomeric *P* element—that is, they were similar to the excision frequency of the M cytotype control. Thus, each of the *aub* alleles utterly abolished repression of *P*-element excision by the P cytotype. The other mutations that were tested—three alleles of *hls* and two alleles of *piwi*—did not impair this repression, at least in heterozygous condition. Unfortunately, the sterility and lethality associated with these mutations prevents an assessment of their homozygous effects on cytotype-mediated repression.

Disruption of cytotype-mediated repression in mutant *aub* stocks: The abolition of cytotype-mediated repression of *P* excisions by the *aub* mutations was investigated more fully in two additional experiments. One experiment assessed *P*-excision frequencies in the male germline and the other assessed these frequencies in the female germline. Both experiments were initiated by crossing *TP* sn^w; *aub*/*Cy Roi* females to *H(hsp/CP)2* males. In the first experiment, the *TP* sn^w; *aub*/*H(hsp/CP)2* sons were crossed to attached-*X* females and, in the second experiment, the *TP* sn^w/+; *aub*/*H(hsp/CP)2* daughters were crossed to sn^3 males. The offspring from these two types of crosses provided data on the occurrence of *P* excisions in the parental germlines. For the females, only excisions leading to extreme singed offspring could be detected, whereas for the males, excisions producing either extreme singed or wild-type offspring were identifiable. In each experiment, flies that inherited the *Cy Roi* balancer chromosome instead of the mutant *aub* chromosome were also tested. Data from these flies made it possible to ascertain if the *aub* mutations exerted a maternal effect on repression of *P*-element excisions. In addition, to test if the *aub* mutations affected the frequency of *P* excisions *per se*, flies from mutant stocks that did not carry a telomeric *P* element were analyzed in both experiments.

Table 3 presents the results of the experiment to study *P*-excision frequencies in males. In the absence of either a telomeric *P* element or an *aub* mutation, the *P*-excision frequency was 0.459, which is similar to the excision frequency of the M cytotype control in Table 2. Among flies that carried *TP5*, this frequency was reduced to 0.020, and among flies that carried *TP6*, it was reduced to 0.154. Thus, as expected, both telomeric *P* elements repressed *P* excisions from sn^w significantly. However, this repression was profoundly disrupted by each of the *aub* mutations. *TP5* and *TP6* males that carried either of these mutations had excision frequencies similar to or greater than the control excision frequency of 0.459. Furthermore, their brothers, which carried the *Cy Roi* balancer chromosome instead of the mutant *aub* chromosome, also showed high excision frequencies. Thus, disruption of cytotype-mediated repression of *P* excisions by the *aub* mutations appears to involve a maternal effect; *TP5* or *TP6* males whose mothers were heterozygous for an *aub* mutation could not repress *P* excisions, even when they did not inherit the *aub* mutation itself.

This experiment also provided information on the effect of the *aub* mutations on the frequency of *P* excisions in flies lacking telomeric *P* elements. These frequencies ranged from 0.495 to 0.614, and three of

TABLE 3

Maternal effect of mutations in the *aubergine* gene on cytotype-mediated repression of *P* excisions from *sn^w* in the male germline

		Non-Curly sons tested[a]			Curly sons tested[a]		
TP	Mother's genotype	No. of vials	No. of flies	Excision rate ± SE[b]	No. of vials	No. of flies	Excision rate ± SE[b]
None	+/+	30	1268	0.459 ± 0.014	—	—	—
None	$aub^{\Delta P\text{-}3a}$/*Cy Roi*	33	1114	0.593 ± 0.024	27	1027	0.614 ± 0.031
None	aub^{QC42}/*Cy Roi*	20	745	0.495 ± 0.024	18	665	0.564 ± 0.025
TP5	+/+	30	1523	0.020 ± 0.006	—	—	—
TP5	$aub^{\Delta P\text{-}3a}$/*Cy Roi*	20	909	0.530 ± 0.032	19	827	0.546 ± 0.028
TP5	aub^{QC42}/*Cy Roi*	29	996	0.436 ± 0.038	25	892	0.420 ± 0.034
TP6	+/+	29	1549	0.154 ± 0.018	—	—	—
TP6	$aub^{\Delta P\text{-}3a}$/*Cy Roi*	25	1288	0.567 ± 0.026	24	1183	0.521 ± 0.027
TP6	aub^{QC42}/*Cy Roi*	29	1426	0.594 ± 0.023	21	998	0.556 ± 0.023

[a] The sons were heterozygous for the *H(hsp/CP)2* transgene, which encodes the P transposase. Phenotypically non-Curly sons were (*TP*) *sn^w*; *aub* or +/*H(hsp/CP)2* and phenotypically Curly sons were (*TP*) *sn^w*; *Cy Roi*/*H(hsp/CP)2*; that is, they did not carry an *aub* mutation.
[b] Average unweighted excision rate $[(sn^+ + sn^e)/(sn^w + sn^+ + sn^e)]$ ± standard error.

them were significantly greater than the control frequency of 0.459. These higher frequencies suggest that an *aub* mutation in the mother's genotype actually enhances the occurrence of *P* excisions, even when the *aub* mutation is not inherited by the offspring. This effect is particularly notable for the $aub^{\Delta P\text{-}3a}$ allele, which was associated with a 30% increase in the frequency of *P* excisions. As a check on the possibility that mutations in the *hls* and *piwi* genes might also increase the *P*-excision frequency, we tested *sn^w* males that were heterozygous for these mutations and the *H(hsp/CP)2* transgene, but that did not carry a telomeric *P* element—that is, that had the M cytotype. The data, shown in supplemental Table S1 (http://www.genetics.org/supplemental/), indicate that none of the tested mutations had a significant effect on the frequency of *P* excisions from *sn^w*.

Table 4 presents the results of the experiment used in studying the effect of the *aub* mutations on cytotype-mediated repression of *P* excisions in females. These excision frequencies are not comparable to those obtained from males because only one class of *P* excisions could be detected. Furthermore, only one telomeric *P* element (*TP5*) was studied in this experiment. The results show that *TP5* strongly repressed *P* excisions in the female germline and that each of the *aub* mutations disrupted this repression profoundly. Moreover, as in the experiment with males, the *aub* mutations disrupted *TP5*-mediated repression through a maternal effect. Also, as in the experiment with males, the $aub^{\Delta P\text{-}3a}$ allele was associated with a dramatic increase in the frequency of *P* excisions from *sn^w*. Three of the four groups of flies involving this allele had excision frequencies significantly

TABLE 4

Maternal effect of mutations in the *aubergine* gene on cytotype-mediated repression of *P* excisions from *sn^w* in the female germline

		Non-Curly daughters tested[a]			Curly daughters tested[a]		
TP	Mother's genotype	No. of vials	No. of flies	Excision rate ± SE[b]	No. of vials	No. of flies	Excision rate ± SE[b]
None	+/+	27	1515	0.122 ± 0.013	—	—	—
None	$aub^{\Delta P\text{-}3a}$/*Cy Roi*	22	979	0.227 ± 0.025	22	643	0.228 ± 0.021
None	aub^{QC42}/*Cy Roi*	26	1054	0.169 ± 0.017	28	895	0.174 ± 0.018
TP5	+/+	23	1136	0.004 ± 0.002	—	—	—
TP5	$aub^{\Delta P\text{-}3a}$/*Cy Roi*	29	1087	0.240 ± 0.026	23	841	0.171 ± 0.029
TP5	aub^{QC42}/*Cy Roi*	29	1476	0.166 ± 0.023	26	1083	0.162 ± 0.022

[a] The daughters were heterozygous for the *H(hsp/CP)2* transgene, which encodes the P transposase. Phenotypically non-Curly daughters were (*TP5*) *sn^w*/+; *aub* or +/*H(hsp/CP)2* and phenotypically Curly daughters were (*TP5*) *sn^w*/+; *Cy Roi*/*H(hsp/CP)2*; that is, they did not carry an *aub* mutation.
[b] Average unweighted excision rate $[sn^e/(sn^w + sn^e)]$ ± standard error.

TABLE 5

Effects of paternally inherited *aubergine* mutations on cytotype-mediated repression of *P* excisions from sn^w in the male germline

TP	Father's genotype	Non-Curly sons tested[a] No. of vials	No. of flies	Excision rate ± SE[b]	Curly sons tested[a] No. of vials	No. of flies	Excision rate ± SE[b]
None	+/+	30	1030	0.377 ± 0.018	—	—	—
None	$piwi^1$/*Cy Roi*	29	756	0.402 ± 0.022	30	877	0.398 ± 0.024
None	$aub^{\Delta P\text{-}3a}$/*Cy Roi*	30	930	0.427 ± 0.020	30	962	0.445 ± 0.021
None	aub^{QC42}/*Cy Roi*	30	1069	0.464 ± 0.022	30	1009	0.571 ± 0.019
TP5	+/+	30	782	0.028 ± 0.010	—	—	—
TP5	$piwi^1$/*Cy Roi*	29	825	0.016 ± 0.007	29	822	0.039 ± 0.009
TP5	$aub^{\Delta P\text{-}3a}$/*Cy Roi*	29	703	0.016 ± 0.009	30	858	0.057 ± 0.013
TP5	aub^{QC42}/*Cy Roi*	30	1155	0.009 ± 0.005	29	1076	0.034 ± 0.006

[a] The sons were heterozygous for the *H(hsp/CP)3* transgene, which encodes the P transposase. Phenotypically non-Curly sons were (*TP5*) sn^w; mutation/+; *H(hsp/CP)3*/+ or (*TP5*) sn^w; +/+; *H(hsp/CP)3*/+, and phenotypically Curly sons were (*TP5*) sn^w; *Cy Roi*/+; *H(hsp/CP)3*/+; that is, they did not carry an *aub* or a *piwi* mutation.

[b] Average unweighted excision rate $[(sn^+ + sn^e)/(sn^w + sn^+ + sn^e)]$ ± standard error.

greater—in fact, nearly two times greater—than the control frequency of 0.122.

Determining when *aub* mutations disrupt the P cytotype: To ascertain if *aub* mutations act zygotically to disrupt the P cytotype, we crossed *aub/Cy Roi* males that were also homozygous for the *H(hsp/CP)3* transgene inserted on chromosome *3* to sn^w females. One group of these females was homozygous for *TP5* (and therefore had the P cytotype) whereas the other group lacked this telomeric *P* element (and therefore had the M cytotype). The sn^w; *aub*/+; *H(hsp/CP)3*/+ sons and sn^w/+; *aub*/+; *H(hsp/CP)3*/+ daughters from these crosses were then tested for *P* excisions from sn^w. We also tested their sn^w; *Cy Roi*/+; *H(hsp/CP)3*/+ and sn^w/+; *Cy Roi*/+; *H(hsp/CP)3*/+ siblings. As controls, we tested flies that did not have an *aub* mutation in the genotype, and we also tested flies that had the $piwi^1$ mutation in place of the *aub* mutation. The results from all these tests are shown in Table 5 (males) and Table 6 (females).

Neither sex shows evidence of disruption of *TP5*-mediated repression by a zygotic effect of the *aub* mutations. Compared to the M cytotype controls, the flies that carried *TP5* had low *P*-excision frequencies, regardless of genotype. Thus, the P cytotype associated with the *TP5* element is not immediately disrupted by the zygotic effect of a paternally inherited *aub* mutation either in males or in females.

These results imply that the *aub* mutations require more than one generation to disrupt *TP5*-mediated regulation of *P* excisions. To see if this disruption could

TABLE 6

Effects of paternally inherited *aubergine* mutations on cytotype-mediated repression of *P* excisions from sn^w in the female germline

TP	Father's genotype	Non-Curly daughters tested[a] No. of vials	No. of flies	Excision rate ± SE[b]	Curly daughters tested[a] No. of vials	No. of flies	Excision rate ± SE[b]
None	+/+	28	937	0.101 ± 0.012	—	—	—
None	$piwi^1$/*Cy Roi*	27	932	0.126 ± 0.018	30	637	0.124 ± 0.019
None	$aub^{\Delta P\text{-}3a}$/*Cy Roi*	28	1148	0.096 ± 0.012	28	962	0.120 ± 0.015
None	aub^{QC42}/*Cy Roi*	29	1019	0.143 ± 0.017	26	860	0.151 ± 0.017
TP5	+/+	30	1405	0.005 ± 0.003	—	—	—
TP5	$piwi^1$/*Cy Roi*	28	1093	0.002 ± 0.002	24	1160	0.003 ± 0.002
TP5	$aub^{\Delta P\text{-}3a}$/*Cy Roi*	30	1214	0.003 ± 0.001	24	892	0.004 ± 0.002
TP5	aub^{QC42}/*Cy Roi*	13	475	0.024 ± 0.010	15	449	0.023 ± 0.008

[a] The daughters were heterozygous for the *H(hsp/CP)3* transgene, which encodes the P transposase. Phenotypically non-Curly daughters were (*TP5*) sn^w /+; mutation/+; *H(hsp/CP)3*/+ or (*TP5*) sn^w/+; +/+; *H(hsp/CP)3*/+, and phenotypically Curly daughters were (*TP5*) sn^w/+; *Cy Roi*/+; *H(hsp/CP)3*/+; that is, they did not carry an *aub* or a *piwi* mutation.

[b] Average unweighted excision rate $[sn^e/(sn^w + sn^e)]$ ± standard error.

TABLE 7

Effects of grandpaternally inherited *aubergine* mutations on cytotype-mediated repression of *P* excisions from sn^w in the male germline

Mother's genotype[a]	Non-Curly sons tested			Curly sons tested		
	No. of vials	No. of flies	Excision rate ± SE[b]	No.of vials	No. of flies	Excision rate ± SE[b]
w sn^w/+; $piwi^1$/*Cy Roi*	25	747	0.518 ± 0.020	29	837	0.583 ± 0.021
w sn^w/+; $aub^{\Delta P\text{-}3a}$/*Cy Roi*	20	445	0.500 ± 0.031	21	489	0.587 ± 0.031
w sn^w/+; aub^{QC42}/*Cy Roi*	23	722	0.495 ± 0.026	25	751	0.571 ± 0.025
TP5 w sn^w/+; $piwi^1$/*Cy Roi*	29	393	0.216 ± 0.031	30	343	0.287 ± 0.037
TP5 w sn^w/+; $aub^{\Delta P\text{-}3a}$/*Cy Roi*	28	732	0.608 ± 0.032	27	398	0.512 ± 0.040
TP5 w sn^w/+; aub^{QC42}/*Cy Roi*	25	614	0.461 ± 0.030	29	782	0.406 ± 0.033

[a] These flies were created by crossing *w* sn^w; $piwi^1$/*Cy Roi* or *TP5 w* sn^w; $piwi^1$/*Cy Roi* females to +; mutation/*CyO* males, where the mutation was $piwi^1$, $aub^{\Delta P\text{-}3a}$, or aub^{QC42}. They were crossed to males homozygous for the *H(hsp/CP)2* transgene, and their non-Curly and Curly sons that had orange (rather than red) eyes and weak singed (rather than wild-type) bristles—that is, that carried the *w* and sn^w alleles on the *X* chromosome and the *H(hsp/CP)2* transgene on chromosome *2*—were tested for *P* excisions. Because the *w* mutation is tightly linked to the left *X* telomere, it could be used as a marker for the presence of *TP5*. The non-Curly sons were (*TP5*) *w* sn^w; mutation/*H(hsp/CP)2*; that is, they carried the *aub* or *piwi* mutation, whereas the Curly sons, which were (*TP5*) *w* sn^w; *Cy Roi*/*H(hsp/CP)2*, did not.

[b] Average unweighted excision rate $[(sn^+ + sn^e)/(sn^w + sn^+ + sn^e)]$ ± standard error.

occur within two generations, we tested the effects of *aub* mutations on repression of *P* excisions in the grandsons of P cytotype *TP5 w* sn^w females. Flies carrying the $piwi^1$ mutation, which does not disrupt the P cytotype, were used as controls in this experiment. The test males were the sons of F_1 females that were contrived to be heterozygous for the *TP5 w* sn^w *X* chromosome, which was maternally inherited, and the $piwi^1$ or *aub* mutation, which was paternally inherited. These females, which also carried a maternally inherited *Cy Roi* balancer chromosome, were crossed to males homozygous for the *H(hsp/CP)2* transgene to obtain the males for the excision tests. For comparison, we also measured the frequency of *P* excisions in males derived in a similar way from M cytotype *w* sn^w grandmothers. The results of all these tests are presented in Table 7 along with details of the genetic manipulations.

The M-cytotype-derived flies that carried the $piwi^1$ or *aub* mutations had *P*-excision frequencies of ∼0.50. Their siblings, which carried the *Cy Roi* balancer chromosome instead of the $piwi^1$ or *aub* mutant chromosome, had higher excision frequencies of ∼0.58. Thus, in the M cytotype, the balancer chromosome appears to elevate the *P*-excision rate somewhat. The P-cytotype-derived flies that carried the $piwi^1$ mutation had an excision frequency of 0.216, and their *Cy Roi* siblings had a frequency of 0.287. These frequencies indicate some repression of *P* excision, albeit not as much as in the sons (rather than the grandsons) of P cytotype females (excision frequency = 0.02–0.04; see Table 5). In a two-generation experiment, however, some repression ability is expected to be lost because the *TP5* element is not homozygous in the mothers of the tested males (Niemi *et al.* 2004). Other data in Table 7 indicate that the *aub* mutations exacerbate this loss significantly. The P-cytotype-derived flies that carried the *aub* mutations had *P*-excision frequencies of 0.60 ($aub^{\Delta P\text{-}3a}$) and 0.46 (aub^{QC42}), and their *Cy Roi* siblings had excision frequencies of 0.51 and 0.40, respectively. These high excision frequencies—similar to those observed in the M cytotype controls—indicate that cytotype regulation by a telomeric *P* element is profoundly disrupted by *aub* mutations through a maternal effect.

Assessing the persistence of cytotype disruption by *aub* mutations: Mutations in the *Su(var)205* gene disrupt the P cytotype for several generations after they have been removed from the genotype of a stock homozygous for the *TP5* element. The persistence of this disruption is thought to involve the elongation of telomeres in stocks heterozygous for a *Su(var)205* mutation (Haley *et al.* 2005). To see if *aub* mutations might have a similar effect, we extracted *X* chromosomes from *TP5* sn^w; *aub*/*Cy Roi* stocks and made them homozygous in the absence of the *aub* mutation. Each of the resulting homozygous *TP5* sn^w lines was then assayed for *P* excisions by crossing females from them to *H(hsp/CP)2* males and then crossing the *TP5* sn^w; *H(hsp/CP)2*/+ sons to attached-*X* females. As controls, we carried out a parallel analysis of *X* chromosomes extracted from a *TP5* sn^w; $piwi^1$/*Cy Roi* stock in which cytotype regulation is intact. The results of all these tests are shown in Table 8.

To gauge the effectiveness of repression by the lines tested in this experiment, we measured the frequency of *P* excisions occurring in sn^w flies that came from the standard M cytotype sn^w stock. Among 29 such flies, the

TABLE 8

Repression of *P* excisions from sn^w by lines homozygous for *TP5* sn^w *X* chromosomes extracted from mutant *aubergine* and *piwi* stocks

Original mutation	Line[a]	No. of vials	No. of flies	Excision rate ± SE[b]
$piwi^1$	1	29	973	0.017 ± 0.005
	2	28	855	0.032 ± 0.015
	3	28	895	0.080 ± 0.028
	4	26	814	0.052 ± 0.009
	5	29	926	0.008 ± 0.005
	6	28	940	0.172 ± 0.028
	7	22	658	0.012 ± 0.007
	8	27	839	0.009 ± 0.004
$aub^{\Delta P\text{-}3a}$	1	26	813	0.029 ± 0.012
	2	30	939	0.056 ± 0.013
	3	25	761	0.030 ± 0.013
	4	29	1009	0.020 ± 0.011
	5	25	726	0.444 ± 0.050
	6	29	875	0.019 ± 0.008
	7	25	807	0.022 ± 0.010
	8	25	906	0.004 ± 0.003
aub^{QC42}	1	30	930	0.047 ± 0.011
	2	22	511	0.011 ± 0.006
	3	24	766	0.049 ± 0.016
	4	24	843	0.075 ± 0.015
	5	27	878	0.020 ± 0.008
	6	26	832	0.059 ± 0.017
	7	28	824	0.023 ± 0.010
	8	25	446	0.042 ± 0.019

[a] The lines were obtained by crossing individual males from each mutant stock to attached-*X* females. A single *TP5* sn^w; *Cy Roi*/+ son from each cross was backcrossed to attached-*X* females to purge the line of the *aub* or *piwi* mutation. *TP5* sn^w; +/+ sons from these backcrosses were then double mated, first to attached-*X* females and then to *FM7*/sc^7 *l* females. From the latter mating, *TP5* sn^w/*FM7* daughters were selected and crossed to *TP5* sn^w sons from the former mating to obtain homozygous *TP5* sn^w daughters and hemizygous *TP5* sn^w sons, which were then intercrossed to establish a line. Granddaughters of these intercrosses were used to initiate the tests reported here. The tested males were *TP5* sn^w; *H(hsp/CP)2*/+.

[b] Average unweighted excision rate $[(sn^+ + sn^e)/(sn^w + sn^+ + sn^e)] \pm$ standard error.

average excision rate was 0.464 ± 0.022. We also tested flies that came from the standard P cytotype *TP5* sn^w stock; among 29 of these flies, the average excision rate was 0.015 ± 0.010.

Eight lines were derived independently from each of the *TP5* sn^w; $aub^{\Delta P\text{-}3a}$/*Cy Roi*, *TP5* sn^w; aub^{QC42}/*Cy Roi*, and *TP5* sn^w; $piwi^1$/*Cy Roi* stocks. Among these 24 lines, only 2 showed marked impairment of cytotype-mediated repression of *P* excisions from sn^w. The excision rate for line 5 from the *TP5* sn^w; $aub^{\Delta P\text{-}3a}$/*Cy Roi* stock was 0.444—similar to that of the M cytotype control—and the rate for line 6 from the *TP5* sn^w; $piwi^1$/*Cy Roi* stock was 0.172. All the other excision rates were <0.08. Thus, in the vast majority of the lines, including 15 of the 16 lines derived from the mutant *aub* stocks, cytotype regulation was intact. These results indicate that, unlike *Su(var)205* mutations, *aub* mutations do not generally disrupt cytotype regulation several generations after they have been purged from the genotype.

To see if the *TP5* element was still present in the two anomalous lines, we used the polymerase chain reaction. For each line, DNA was obtained separately from five males that had been reserved from the testcrosses. These DNA samples were then used to seed a PCR that specifically amplifies the *TP5* element; see Stuart *et al.* (2002) for a description of the *TP5*-specific primer and the PCR procedure. The results indicated that *TP5* was present in each of the testcross males. Thus, the high excision rates of the two anomalous lines were not due to the loss of *TP5* during the genetic manipulations that led to the lines. Rather, some other phenomenon must account for their inability to repress *P* excisions effectively.

DISCUSSION

Our data indicate that the *aubergine* gene plays an important role in cytotype regulation of the *P*-element family. Two mutations that were independently induced in this gene disrupted repression of *P*-element excision in the germline through heterozygous effects in females that carried *X*-linked telomeric *P* elements. These effects were manifested in both the sons and the daughters of heterozygous mutant females, whether or not they inherited the *aub* mutation itself. However, these same *aub* mutations, when paternally inherited, had no effect on the cytotype system of *P*-element repression. These results imply that the *aubergine* gene product is needed to establish and maintain the P cytotype in the female germline. Moreover, this product is apparently needed in quantity because cytotype regulation is compromised by simply depleting—not eliminating—the genes encoding this protein in the maternal germline. Mutations in two other RNAi genes, *piwi* and *homeless*, did not have effects on *P*-element regulation. However, these negative results do not exclude *piwi* and *hls* from influencing cytotype because our experiments were limited to tests for heterozygous effects. A mutation in a fourth gene, *Enhancer of zeste*, which had been implicated in cytotype regulation by Roche and Rio (1998) by experiments that were subsequently questioned (Rio 1999), also had no effect on repression of *P*-element excision.

Disruption of cytotype regulation by heterozygous *aub* mutations suggests that in the germline *P* elements are controlled by an RNAi mechanism. Other investigations have shown that cytotype regulation is associated with *P* elements inserted in the TAS at the left end of the *X* chromosome (Ronsseray *et al.* 1991; Marin *et al.* 2000; Stuart *et al.* 2002) and that these elements interact synergistically with *P* elements scattered throughout the genome to bring about strong repression of the *P*-element family (Simmons *et al.* 2007, accompanying article in this

issue). Moreover, this repression appears to be mediated by products of the telomeric *P* elements—presumably RNAs, because neither of the telomeric *P* elements studied here seems to encode a polypeptide with significant repression ability (Stuart *et al.* 2002; P. Jensen, J. Stuart, M. Goodpaster, K. Newman, J. Goodman and M. Simmons, unpublished results). Marin *et al.* (2000) also documented repression by a *P* element unlikely to produce a repressor polypeptide.

A plausible model is that telomeric *P* elements are transcribed in both directions to produce double-stranded RNA, which then induces RNAi to silence *P* elements throughout the genome. The RNAi response may be intensified if other nontelomeric *P* elements also contribute to the formation of double-stranded RNA. For *TP5* and *TP6*, sense transcripts could be produced by transcription from the *P*-element promoter or, because both of these elements are oriented toward the interior of the chromosome, by readthrough transcription from the retrotransposon array at the chromosome's end. Antisense transcripts of these elements could be produced by transcription from an outward-directed promoter located on the 3′ side of the telomeric *P* element, possibly somewhere in the TAS. The amount of double-stranded *P* RNA that could form would therefore depend on the relative strengths of these opposing transcriptional efforts. Once formed, double-stranded *P* RNA could be diced into small interfering RNAs, which could repress *P*-element activity either by inducing the degradation of transposase mRNA or by altering chromatin structure around *P* elements throughout the genome. These small interfering RNAs could also be transmitted through eggs to silence *P* activity in the next generation. Experiments using molecular techniques are needed to test these ideas.

There are, however, reasons to believe that this model is correct in its broad outline. Savitsky *et al.* (2006) have reported that the retrotransposons at the tips of Drosophila chromosomes are under the control of an RNAi mechanism. Insertion of these retrotransposons at the ends of chromosomes normally replenishes sequences lost by the asymmetry of DNA replication there (Biessmann *et al.* 1990; Mason and Biessmann 1995). However, mutations in *aub* and *hls* allow the retrotransposons to insert more frequently than they otherwise would, ultimately producing longer telomeres (Savitsky *et al.* 2006). This process of telomere elongation is germline specific and appears to be mediated by sense transcripts of the telomeric retrotransposons, which accumulate in the germlines of *aub* and *hls* mutant females, evidently because the *aub* and *hls* mutations impair a regulatory system that is based on RNAi. Cytotype regulation by telomeric *P* elements may use the same RNAi system. In fact, this regulation may simply be an inadvertent consequence of *P* elements having inserted into a region whose overall structure is controlled by an RNAi mechanism. Disruption of this mechanism would, therefore, remove a constraint on *P*-element activity in the germline.

In another vein, Vagin *et al.* (2006) have studied the involvement of RNAi in the regulation of the *X*-linked *Stellate* genes by the *Y*-linked *Suppressor of Stellate* locus and the expression of several different retrotransposons, including *HeT-A*, which is telomere specific. All these genomic elements appear to be controlled by an RNAi system that is mediated by repeat-associated small interfering RNAs (rasiRNAs), 24–29 nucleotides long. It is significant that the rasiRNAs appear to bind to the Piwi and Aub proteins in ovaries. Small interfering *P* RNAs might therefore be conveyed from mother to offspring by being bound to either or both of these proteins in eggs.

Cytotype regulation can also be disrupted by mutations in the *Su(var)205* gene (Ronsseray *et al.* 1996), which encodes HP1, a protein involved in chromatin organization (Eissenberg *et al.* 1990). This protein also appears to provide a capping function at the very ends of chromosomes (Fanti *et al.* 1998; Perrini *et al.* 2004). The depletion of HP1 that occurs in stocks heterozygous for a *Su(var)205* mutation allows retrotransposons to attach frequently to chromosome ends (Savitsky *et al.* 2002). When this high level of attachment occurs, the telomeres become elongated. Telomere elongation also occurs in stocks carrying the *Tel* mutation (Siriaco *et al.* 2001); however, the underlying mechanism is unknown. Stocks in which the telomeres have been elongated because *Su(var)205* or *Tel* mutations have been present show impaired cytotype regulation (Ronsseray *et al.* 1996; Haley *et al.* 2005). Haley *et al.* (2005) speculated that this impairment is due to affinities among elongated telomeres that prevent pairing between telomeric *P* elements and other *P* elements in the genome. However, given the evidence for a *bona fide* "cytoplasmic" component of cytotype regulation (Simmons *et al.* 2007, accompanying article in this issue), physical contact between telomeric and other *P* elements is not needed to repress *P* activity. The impaired cytotype that is characteristic of mutant *Su(var)205* and *Tel* stocks may therefore be a consequence of the altered expression of telomeric *P* elements caused by elongated telomeres in these stocks. Additional retrotransposons at chromosome ends enhance transcription of *P* transgenes inserted in the TAS (Golubovsky *et al.* 2001). They may also enhance the transcription of *P* elements inserted in these regions. If the enhanced transcription strongly favors the production of one type of *P* RNA—sense, for example—then the formation of double-stranded RNA could be impaired and the RNAi mechanism it normally induces would be weakened.

One important difference between the effects of *aub* and *Su(var)205* mutations is that *aub* mutations generally seem to impair cytotype regulation only in the short term, whereas *Su(var)205* mutations impair it many generations after they have been purged from the genotype

(Haley *et al.* 2005). At first glance, this difference seems difficult to explain because both types of mutations cause telomere elongation, which is a genetic change that might persist for several generations. However, Savitsky *et al.* (2006) noted that the telomeres were not detectably elongated in the mutant *aub* stock that they studied. Thus, telomere elongation may be less effective in mutant *aub* stocks than in mutant *Su(var)205* stocks, and the impairment of cytotype by *aub* mutations may have more to do with a dysfunctional system for transporting rasiRNAs through oocytes than with a failure to produce these RNAs because the expression of a telomeric *P* element has been altered by adding retrotransposons to the end of the chromosome.

Telomeric *P* elements seem to be common in natural populations (Ajioka and Eanes 1989), possibly because selection has favored their abilities to repress hybrid dysgenesis. These elements can interact with other *P* elements, probably at the level of their products, to repress dysgenesis strongly. Whether nontelomeric *P* elements have the ability to bring about the P cytotype is, at this time, an open question. However, Ronsseray *et al.* (2001) have observed cytotype-like repression associated with clusters of nontelomeric *P* transgenes. Thus, a telomeric *P* element may not be absolutely essential for the P cytotype to develop.

One indication that nontelomeric *P* elements might be capable of initiating regulation by an RNAi mechanism is that *aub* mutations, in particular $aub^{\Delta P\text{-}3a}$, appear to enhance the mutability of sn^w in flies that do not carry a telomeric *P* element (see Tables 3 and 4). This finding could be a result of mutational disruption of an RNAi response initiated by double-stranded *P* RNA transcribed from the two *P* elements inserted in the sn^w allele. These *P* elements are inserted in a head-to-head orientation 8 bp apart in the 5′ region of the *singed* gene. Furthermore, because their sequences are included within some *singed* transcripts (Paterson *et al.* 2007), they could possibly generate double-stranded *P* RNA, which in turn could stimulate an RNAi response to other *P* RNAs, including the P transposase mRNA. By impairing this response, *aub* mutations might increase the likelihood that *P* mRNA will be translated into the P transposase in sn^w flies, leading to an increased frequency of *P*-element excisions from sn^w. Other double-*P* insertions in the *singed* gene have been identified (Eggleston 1990). If double-*P* insertions are common in natural populations, and if they are transcribed, they might trigger RNAi-based mechanisms that regulate *P*-element activity.

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