Cytotype regulation by telomeric *P* elements in *Drosophila melanogaster*: variation in regulatory strength and maternal effects

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(*Received 10 July 2009 and in revised form 24 August 2009*)

Summary

Strains carrying the X-linked telomeric *P* elements *TP5* or *TP6* varied in their ability to repress hybrid dysgenesis. The rank ordering of these strains was consistent across different genetic assays and was not related to the type of telomeric *P* element (*TP5* or *TP6*) present. Strong repression of dysgenesis was associated with weak expression of mRNA from the telomeric *P* element and also with a reduced amount of mRNA from a transposase-producing *P* element contained within a transgene inserted on an autosome. A strictly maternal component of repression, transmitted independently of the telomeric *P* element, was detected in the daughters but not the sons of females from the strongest repressing strains. However, this effect was seen only when dysgenesis was induced by crossing these females to males from a P strain, not when it was induced by crossing them to males homozygous for a single transposase-producing *P* element contained within a transgene. These findings are consistent with the hypothesis that the P cytotype, the condition that regulates *P* elements, involves an RNA interference mechanism mediated by piRNAs produced by telomeric *P* elements such as *TP5* and *TP6* and amplified by RNAs produced by other *P* elements.

1. Introduction

Transposable elements are important constituents of the genomes of most organisms. When they become active, they can cause deleterious mutations and chromosome breaks. It therefore stands to reason that mechanisms would have evolved to prevent or limit their activity. Recent studies have pointed to the phenomenon of RNA-mediated silencing as one of these mechanisms (Blumenstiel & Hartl, 2005; Brennecke *et al.*, 2007, 2008; Josse *et al.*, 2007; Das *et al.*, 2008; Jensen *et al.*, 2008).

In the fruit fly *Drosophila melanogaster*, RNA-mediated silencing has been implicated in the regulation of different kinds of transposable elements, including the *I* factor, which is a retrotransposon, and the *P* element, which is a cut-and-paste transposon (Reiss *et al.*, 2004; Dramad *et al.*, 2007; Simmons *et al.*, 2007; Brennecke *et al.*, 2008; Chambeyron *et al.*,

2008). Both of these transposable elements were discovered through their involvement in a syndrome of abnormal traits in the offspring of crosses between different strains. This syndrome, called hybrid dysgenesis, usually occurs in the offspring of only one of the two reciprocal crosses (Kidwell *et al.*, 1977; Bregliano *et al.*, 1980; Engels, 1989). In the offspring of the other cross, dysgenesis is repressed by a maternally transmitted condition now thought to depend on small RNA molecules that interfere with the expression or functioning of transposon RNA.

In this paper, we focus on the repression of *P*-element-induced hybrid dysgenesis. This type of dysgenesis is restricted to the germ line because the transposase that mobilizes *P* elements is naturally made only in germ-line cells. *P*-induced dysgenesis is manifested by the frequent occurrence of mutations and by the failure of the gonads to develop. This latter effect, called gonadal dysgenesis (GD), is a profound abnormality that causes the flies to be sterile. *P*-induced dysgenesis can be repressed by a maternally inherited condition called the P cytotype (Engels, 1979*a*). A series of studies has tied this repressing

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condition to *P* elements that have fortuitously inserted in the telomere associated sequences (TAS) at the left end of the X chromosome (Ronsseray *et al.*, 1991, 1993, 1996, 1998; Marin *et al.*, 2000; Stuart *et al.*, 2002; Niemi *et al.*, 2004; Simmons *et al.*, 2004; Jensen *et al.*, 2008). This locus is known to produce small RNA molecules that interact with proteins of the PIWI family, and for that reason, its RNA products are called PIWI-interacting, or piRNAs (Brennecke *et al.*, 2007). Recent studies have suggested that piRNAs are the physical basis of the P cytotype (Brennecke *et al.*, 2008). However the mechanistic details, including how these RNAs are produced and act, remain to be elucidated.

Several *P* elements inserted in the TAS at the left end of the X chromosome have been isolated and analysed. These elements vary in size, sequence and insertion site. They also appear to differ in their ability to evoke the P cytotype – that is, in their ability to repress hybrid dysgenesis. For example, the 1·9 kilobase (kb)-long element *TP6* is reported to be a stronger repressor of GD than the 1·8 kb-long element *TP5*. However, in an assay that measured the frequency of *P*-element excisions from the X-linked *singed* (*sn*) locus, *TP5* was the stronger repressor (Stuart *et al.*, 2002). It is not clear if these differences are due to specific features of the elements or to differences in the genomic DNA around them. *Drosophila* telomeres are dynamic structures, differing one from another in length and sequence composition (Mason & Biessmann, 1995). These differences can be seen within the TAS, where telomeric *P* elements are inserted, and also in the array of retrotransposons that forms the portion of the telomere distal to the TAS. Such differences might influence the ability of sequences within the TAS to produce piRNAs.

Recent studies have suggested that piRNAs – for the *I* factor as well as for the *P* element – are transmitted maternally (Brennecke *et al.*, 2008). These RNAs may therefore explain the cytoplasmic component of transposon regulation that has been documented in some genetic analyses. Specifically, daughters that do not inherit a telomeric *P* element (*TP*) from their heterozygous *TP*/+ mothers repress GD about as effectively as daughters that do inherit such an element (Simmons *et al.*, 2007). Thus repression can be mediated by a strictly maternal effect of the *TP*. However, no such effect is seen when the dysgenic trait studied is *P*-element excision in males (Stuart *et al.*, 2002).

In this paper, we use stocks that contain either *TP5* or *TP6* to address several questions about cytotype regulation. One question is whether variation in repression ability is consistent across different genetic assays. Do telomeric *P* elements that strongly repress increased mutability also strongly repress sterility? Another question is whether variation in repression

ability is due to structural differences among the telomeric *P* elements, or to contextual differences in the telomeric DNA. Does repression depend strictly on the *P* element's structure and sequence, or does it depend on the overall structure of the telomere into which the *P* element is inserted? We also ask if the strength of cytotype regulation is related to *P*-element expression. A telomeric *P* element might be expected to produce piRNAs at the expense of mRNAs, and these piRNAs might be expected to target transposase mRNA for destruction. Is strong *P*-element regulation correlated with weak mRNA production from the telomeric *P* element, and is this regulation associated with a reduction in the amount of transposase mRNA? Finally, we ask what circumstances allow the maternal component of cytotype regulation to contribute to the repression of *P* elements in the offspring of dysgenic crosses.

2. Materials and methods

(i) Drosophila *stocks and husbandry*

Information on the special chromosomes and mutant alleles used in the experiments is available on the Flybase website, in Lindsley & Zimm (1992), or in other references cited in the text. Experimental cultures were reared in vials on a standard cornmeal–molasses–dried yeast medium at 25 °C except where noted.

The X-linked telomeric *P* elements *TP5* and *TP6* were genetically isolated from all the other *P* elements in two wild-type strains in late 1997, as described by Stuart *et al.* (2002). In January 1998, these elements were established in homozygous stocks that also carried the linked markers *w*, *m* and *f*. Secondary stocks were set up from these primary stocks to serve as reserves; thereafter the primary and secondary stocks were maintained independently by mass transfers of adults each generation. In March 1999, homozygous *TP5 w sn^w* and *TP6 w sn^w* stocks were established by recombining X chromosomes from the primary *TP* stocks with an X chromosome carrying the double *P*-element insertion mutation *singed-weak* (*sn^w*), as described by Stuart *et al.* (2002); we refer to these recombinants as the primary *TP sn^w* stocks. In September 2004, following the same procedure, additional *TP sn^w* stocks were established by recombining X chromosomes from the secondary *TP5* and *TP6* stocks with an *sn^w* X chromosome; we refer to these later recombinants as the secondary *TP w sn^w* stocks and denote them as *TP5′ sn^w* and *TP6′ sn^w*. Each of the *TP sn^w* and *TP′ sn^w* stocks was started with a single recombinant X chromosome. In addition to the telomeric *P* elements and the *P* elements inserted in the *singed* gene, these recombinant chromosomes carried a small *P* element tightly linked to the *singed* locus.

This element, near but not within the *singed* gene, has been called the 'unsinged' P element (Roiha *et al.*, 1988).

(ii) GD assay for repression of P-element activity

GD is the most severe manifestation of *P*-element activity in the germ line. To test for repression of this trait, females from a stock or a particular cross were mated to males from the P strain Harwich (Kidwell *et al.*, 1977), which is a strong inducer of dysgenesis, at 21 °C. After 3 days, each mated female was transferred to a fresh culture, which was incubated for 11 days at 29 °C, a temperature that enhances the occurrence of GD. The progeny were transferred to a holding vial and allowed to mature for 2 days, and then as many as 20 of the females of each genotype were scored for the presence or absence of eggs. The procedure was to squash the females between two glass slides in a solution of diluted food colouring, which helps to visualize the eggs. Females without any eggs were scored as having GD; those with at least one egg were scored as normal (non-dysgenic). Repression of P activity was indicated by a low frequency of GD.

(iii) Mutability assay for repression of P-element activity

The *singed-weak* (sn^w) allele causes a mild malformation of the bristles on the adult cuticle. This allele is hypermutable because the two *P* elements inserted in it are frequently excised by the action of the P transposase (Engels, 1979*b*, 1989). Excison of one *P* element produces an allele with a more extreme mutant phenotype (sn^e) and excision of the other *P* element produces an allele with a pseudo-wild phenotype ($sn^{(+)}$). With natural transposase sources, these excisions occur only in the germ line. To detect them, the flies in which the excisions are occurring must be crossed appropriately. Individual sn^w males were crossed to $C(1)DX$ females that had attached-X chromosomes and their sons, which inherited an X chromosome patroclinously, were scored for the three possible bristle phenotypes. The proportion of sn^e and $sn^{(+)}$ flies among those scored was used to estimate the frequency of P excisions in the germ lines of the tested males. Individual $sn^w/+$ females were crossed to sn^3 males and their offspring (both sons and daughters) were scored for the sn^w and sn^e phenotypes. Flies that are heterozygous for sn^3 and an sn^e allele derived from sn^w have the sn^e phenotype and flies that are heterozygous for sn^3 and sn^w have an sn^w phenotype. We did not count the sn^+ flies in these cultures because of the pre-existing sn^+ allele in the tested females. The proportion of sn^e flies among those scored was used to estimate the frequency of

P excisions in the female germ line. Obviously the estimates for P excision frequency in males and females are not equivalent. In all the sn^w mutability experiments, the test cultures were scored on days 14 and 17 after they were established. Repression of P activity was indicated by a low frequency of P excisions.

Three sources of the P transposase were used in the sn^w mutability tests: (1) the $P(ry^+, \triangle 2–3)99B$ transgene inserted on chromosome III (Robertson *et al.*, 1988), hereafter denoted as $\triangle 2–3$, (2) the $H(hsp/CP)2$ transgene inserted on chromosome II (Simmons *et al.*, 2002), hereafter denoted as CP and (3) the Harwich P strain, marked either with w (the standard Harwich strain) or with y and w (a strain that was created by incorporating the y marker into the standard strain through a series of backcrosses). When the $\triangle 2–3$ transgene was used, the test males were crossed to attached-X females from a P strain to repress the bristle mosaicism that is caused by $\triangle 2–3$'s ability to destabilize sn^w outside the germ line (Robertson & Engels, 1989).

(iv) Statistical analyses

The experiments to study repression of GD or sn^w mutability included many replicate cultures of each test group. All the data for an experiment were collected within a 1- or 2-week period. Unweighted mean frequencies of GD and P excisions from sn^w were calculated by treating all replicate measurements equally, and the variances associated with these means were calculated empirically. Statistical differences between groups within experiments were assessed by t- or z-tests using standard errors of the unweighted sample means.

(v) RNA isolation and reverse transcription (RT)-PCR

RNA was isolated from groups of 20 whole, virgin females using TRIZOL (Invitrogen) according to the supplier's instructions. RNA pellets were rehydrated in 20 μl diethylpyrocarbonate (DEPC)-treated water and two 4 μl portions from each sample were removed for analysis. One portion was subjected to RT by the ThermoScript reverse transcriptase (Invitrogen) using an oligo-dT primer in a total volume of 20 μl according to the supplier's instructions. The other portion was added to 16 μl of DEPC-treated water to serve as a non-RT control. After RT was completed, both RT and control samples were treated with 1 μl RNase A (10 mg/ml) to clear them of RNA. The DNA in 1 or 2 μl portions from these samples was then amplified by the PCR in a total volume of 25 μl using appropriate primers and temperature profiles; see Jensen *et al.* (2008) for details. The PCR products were analysed on 1% agarose gels run at 70 volts.

Table 1. *Repression of GD and* sn^w *mutability by primary and secondary* TP sn^w *stocks*

Stock[a]	P strain-induced GD in females			$\triangle$2–3-induced sn^w mutability in males			CP-induced sn^w mutability in males		
	No. of vials	No. of flies	%GD±SE[b]	No. of vials	No. of flies	Rate±SE[c]	No. of vials	No. of flies	Rate±SE[c]
w sn^w	25	400	96·7±1·1	45	1158	0·581±0·025	32	653	0·505±0·029
TP5 sn^w	22	362	19·2±1·7*	48	1371	0·068±0·012*	32	1364	0·003±0·001*
TP5′ sn^w	24	351	90·5±1·8	45	1052	0·551±0·040	30	980	0·391±0·024*
TP6 sn^w	25	471	72·4±2·6*	48	986	0·429±0·029*	31	685	0·158±0·019*
TP6′ sn^w	21	144	44·1±6·7*	46	775	0·160±0·034*	32	702	0·036±0·014*

GD was induced in the daughters of crosses between males from the standard Harwich strain (marked with *w*) and stock females. sn^w mutability was induced in the sons of crosses between $\triangle$2–3 or *CP* males and stock females. With $\triangle$2–3 the initial crosses were at 21 °C and with *CP* they were at 25 °C; however, in both cases, the testcrosses to *C(1)DX* females were carried out at 25 °C. The *CP* sn^w mutability data were collected in conjunction with studies of *TP* and *CP* RNA levels (see Fig. 1). Significant repression of GD or sn^w mutability, relative to the *w* sn^w control, is indicated by an asterisk.
[a] See 'Materials and methods' section for the origin of the primary and secondary *TP sn^w* stocks; the secondary stocks are denoted with primes.
[b] Unweighted average percentage of GD among females ± standard error.
[c] Unweighted average frequency of sn$^{(+)}$ and sn^e sons among all sons ± standard error.

3. Results

(i) *Variation in repression of hybrid dysgenesis*

The primary and secondary *TP sn^w* stocks were tested for repression of GD and sn^w mutability shortly after the secondary stocks were established. The P strain Harwich was used to induce GD, and the $\triangle$2–3 transgene was used to induce sn^w mutability. Three years later, the *TP sn^w* stocks were re-tested for repression of sn^w mutability using the *CP* transgene as a source of the P transposase. A *w sn^w* strain that did not carry a telomeric *P* element was included as a control in each of these experiments. The results are summarized in Table 1.

In the initial tests, three of the four *TP sn^w* strains repressed GD and sn^w mutability significantly. The only non-repressor was *TP5′ sn^w*. However, in the later test this strain showed a moderate, but significant, ability to repress sn^w mutability, possibly because the *CP* transgene produces less transposase than the $\triangle$2–3 transgene (compare the control levels of sn^w mutability in these two experiments; also see Simmons *et al.*, 2002). In all three experiments, the rank order of the four strains was the same: *TP5 sn^w* (strongest repressor), *TP6′ sn^w*, *TP6 sn^w* and *TP5′ sn^w* (weakest repressor). Therefore, the results of the three repression assays are consistent even though they involve different phenotypes (GD and sn^w mutability), transposase sources (P strain, $\triangle$2–3 transgene and *CP* transgene) and sexes (GD in females and sn^w mutability in males), and were carried out at different times.

One possible explanation for the weaker repression ability of the *TP5′ sn^w* and *TP6 sn^w* strains is that the telomeric *P* elements had been lost from some of the

X chromosomes in them. To test this possibility, PCR was used to screen for these *P* elements in 15 males from each strain. Genomic DNA was extracted from each male separately using the procedure of Gloor & Engels (1992) and then *TP5*- and *TP6*-specific primers were used in conjunction with a primer near the 3′ end of the *P* element ('3′-in') to amplify the *TP* DNA; see Stuart *et al.* (2002) for details. A product indicating the presence of the telomeric *P* element was obtained in all the amplification reactions. Thus, the low repression ability of the *TP5′ sn^w* and *TP6 sn^w* strains was not due to the loss of the telomeric *P* element.

(ii) *Molecular correlates of strong and weak repression*

The *TP5 sn^w* and *TP5′ sn^w* strains provided an opportunity to examine strong and weak repression at the molecular level. We used RT-PCR to assess the relative amounts of different mRNAs in flies derived from these strains. Total RNA was extracted from females obtained by crossing each of the strains with males homozygous for the *CP* transgene. These females were sisters of the males that were tested for sn^w mutability (right side of Table 1). The polyadenylated RNA in each extract was reverse transcribed into cDNA, and then pairs of primers were used to amplify samples of the cDNA via PCR. The products from these amplifications were assayed by gel electrophoresis and ethidium bromide staining. Eight independent extracts were prepared and processed from each of the two different genotypes (*TP5 sn^w/+; CP/+* and *TP5′ sn^w/+; CP*). In the gels, a sample from one genotype was placed beside a sample from the other
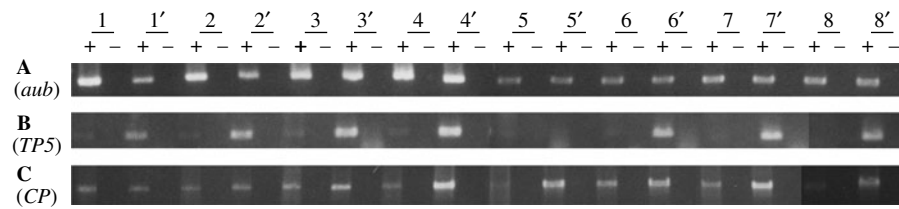
Fig. 1. RT-PCR analysis of germline mRNA from the *TP5 sn^w* (strong repressing) and *TP5' sn^w* (weak repressing) strains. Each strain is represented by eight independently obtained samples, with those from the *TP5' sn^w* strain denoted by a prime. Information on the reagents and conditions for the PCR amplifications is given in Jensen *et al.* (2008). A plus denotes where a sample has been reverse transcribed, and a minus denotes where it has not. (A) Amplification over 25 cycles using primers Aub-d and Aub-u to detect an 848-bp product from *aubergine* mRNA. (B) Amplification over 30 cycles using primers TP5-d and P△2/3-u to detect a 471-bp product from *TP5* germ-line mRNA. (C) Amplification over 30 cycles using primers P0/1-d and P△2/3-u to detect a 1495-bp product from *CP* germ-line mRNA.

to permit pairwise comparisons. Figure 1 presents the results of these RT-PCR experiments.

Panel A in Fig. 1 shows the products obtained with primers specific for *aubergine*, a gene known to be expressed in the female germ line. Among the eight pairwise comparisons, there is no consistent difference between the *TP5 sn^w* and *TP5' sn^w* samples – therefore no consistent difference in *aub* mRNA abundance in the *TP5 sn^w*/+; *CP*/+ and *TP5' sn^w*/+; *CP*/+ genotypes. This result implies that overall the *TP5 sn^w*/+; *CP*/+ and *TP5' sn^w*/+; *CP*/+ extracts contained equivalent amounts of mRNA, and that whatever differences exist among them are due to random variation.

Given the overall equivalence of the two types of samples with respect to total mRNA, we performed experiments to see if the *TP5 sn^w*/+; *CP*/+ and *TP5' sn^w*/+; *CP*/+ genotypes differed systematically with respect to specific *P* mRNAs. Panel B shows the products of RT-PCR with primers specific for germ-line mRNA from the *TP5* element. The specificity of this amplification arises from the fact that one primer straddles the deletion breakpoints in the *TP5* element and the other primer straddles an intron that is removed from *P* RNA only in germ-line cells. Thus, panel B provides information about the abundance of *TP5* RNA exclusively in the germ line, which is the physiologically relevant tissue for studying *P*-element regulation. Only trace amounts of the PCR product were detected in the samples from the *TP5 sn^w*/+; *CP*/+ flies. Significantly more product was detected in seven of the *TP5' sn^w*/+; *CP*/+ samples. Under the assumption of equivalent amounts of mRNA in the two types of samples, this result or a more extreme one should occur with probability 0·035. Thus, by a one-sided paired comparison test, we must reject the hypothesis that the two types of samples contain equivalent amounts of *TP5* mRNA in the germ line. The consistent difference observed between the *TP5 sn^w*/+; *CP*/+ and *TP5' sn^w*/+; *CP*/+ RNA samples indicates that strong repression of *P*-induced hybrid dysgenesis is associated with weak expression of mRNA from the *TP5* element.

Jensen *et al.* (2008) provided evidence that cytotype repression of hybrid dysgenesis involves a reduction in the amount of transposase-encoding *P* mRNA. In the material at hand, this RNA is produced by the *CP* transgene. Panel C in Fig. 1 shows the products of RT-PCR with primers specific for *CP* mRNA in the germ line. In this amplification one primer straddles an intron present in *CP* but not in *TP5*, and the other primer straddles the *P* intron that is removed only in germ-line cells. The binding site for this latter primer is not present in either of the *P* elements inserted in the *sn^w* allele, or in the 'unsigned' element tightly linked to *sn^w*. Thus, DNAs derived from any of these elements are not amplified in reactions with this primer. In seven of the eight pairwise comparisons between samples from the strong and weak repressing genotypes, germ-line *CP* mRNA was less abundant in the strong repressing sample. This statistically significant result indicates that the strength of repression is mechanistically related to a reduction in the amount of transposase-encoding mRNA.

(iii) *Maternal transmission of repression ability*

Cytotype regulation by telomeric *P* elements involves a maternal effect. To investigate this effect, we measured the incidence of dysgenic traits in the offspring of females that were heterozygous for a telomeric *P* element. These females were created by crossing females from the various *TP sn^w* stocks to *y sn^w* males; the *TP sn^w* stocks all carry the *y^+* allele of the *y* locus for body colour. Dysgenesis was then induced by crossing the *TP sn^w*/*y sn^w* F₁ females to Harwich *y w* males. Among the F₂ offspring of these crosses, the presence or absence of the telomeric *P* element was ascertained by following the body colour locus, which is very tightly linked to the left telomere of the X chromosome – and therefore to the *TP* insertion site; the offspring with grey bodies carried the *y^+* allele and the telomeric *P* element and those with yellow bodies did not. By observing the level of dysgenesis in the two types of offspring and in controls created by using the *w sn^w* stock, we could determine if repression

Table 2. *Repression of P strain-induced hybrid dysgenesis in the offspring of* TP sn^w/y sn^w *females that had homozygous* TP sn^w *mothers*

Stock	TP^a	GD in females			sn^w mutability in males			sn^w mutability in females		
		No. of vials	No. of flies	%GD$\pm$SE^b	No. of vials	No. of flies	Rate$\pm$SE^c	No. of vials	No. of flies	Rate$\pm$SE^d
$w\ sn^w$	$-^e$	25	241	98·1$\pm$0·9	32	985	0·333$\pm$0·020	32	1656	0·150$\pm$0·011
	$-^f$	25	213	97·9$\pm$1·3	32	961	0·341$\pm$0·021	26	962	0·171$\pm$0·019
$TP5\ sn^w$	+	19	166	25·7$\pm$5·6*	32	1263	0·028$\pm$0·008*	32	1656	0·012$\pm$0·004*
	−	19	148	28·4$\pm$7·0*	32	1084	0·286$\pm$0·017	32	2387	0·008$\pm$0·003*
$TP5'\ sn^w$	+	22	190	86·1$\pm$4·1*	32	1702	0·108$\pm$0·020*	32	1979	0·088$\pm$0·018*
	−	21	204	95·7$\pm$1·9	32	1096	0·295$\pm$0·017	32	1525	0·151$\pm$0·013
$TP6\ sn^w$	+	25	317	91·1$\pm$2·6*	32	1447	0·154$\pm$0·028*	26	1284	0·077$\pm$0·014*
	−	25	313	98·1$\pm$1·2	32	1196	0·349$\pm$0·020	32	1469	0·136$\pm$0·011
$TP6'\ sn^w$	+	20	136	51·5$\pm$8·4*	32	1475	0·139$\pm$0·019*	32	1414	0·064$\pm$0·015*
	−	19	131	57·1$\pm$8·7*	32	1424	0·318$\pm$0·022	32	1307	0·138$\pm$0·019

The tested offspring were obtained in a two-generation scheme. Stock females were crossed to $y\ sn^w$ males at 25 °C and the TP sn^w/$y\ sn^w$ F$_1$ daughters were then crossed to Harwich $y\ w$ males to induce hybrid dysgenesis. One set of F$_1$ cultures was reared at 29 °C and the resulting F$_2$ females were scored for GD; the females with grey bodies carried the TP, whereas those with yellow bodies did not. A second set of F$_1$ cultures was reared at 21 °C. F$_2$ TP sn^w (grey) and $y\ sn^w$ (yellow) sons from these cultures were individually crossed to $C(1)DX,\ y\ w\ f$ females to produce F$_3$ flies, which were scored to obtain the data on sn^w mutability in the male germ line. F$_2$ TP sn^w/$y\ sn^+$ (grey) and $y\ sn^w$/$y\ sn^+$ (yellow) daughters from these same F$_1$ cultures were individually crossed to sn^3 males to produce F$_3$ flies, which were scored to obtain the data on sn^w mutability in the female germ line. Significant repression of hybrid dysgenesis relative to the corresponding $w\ sn^w$ control is indicated by an asterisk.
^{a} *TP* present (+) or absent (−) in F$_2$ flies.
^{b} Unweighted average percentage GD$\pm$standard error.
^{c} Unweighted average frequency of sn$^{(+)}$ and sn^e sons among all sons$\pm$standard error.
^{d} Unweighted average frequency of sn$^{(e)}$ offspring among sn^w and sn^e offspring$\pm$standard error.
^{e} F$_2$ flies with grey bodies.
^{f} F$_2$ flies with yellow bodies.

was mediated by a strictly maternal effect of the telomeric *P* element. Table 2 summarizes the results of these experiments.

Three dysgenic traits were monitored: GD in females, sn^w mutability in males and sn^w mutability in females. All three traits were repressed in flies that inherited a telomeric *P* element from the heterozygous F$_1$ females, no matter which strain the element came from. This type of repression reflects both maternal and zygotic effects of the telomeric *P* element. The greatest repression by these combined effects was seen in the flies derived from the *TP5* sn^w strain, which was known to be the strongest repressor (Table 1). Much less effective repression was seen in the flies derived from the other strains. Significant repression by a strictly maternal effect was detected only in tests involving females. GD was repressed in females from both *TP5* sn^w and *TP6'* sn^w (the two strongest repressing strains according to Table 1), and sn^w mutability was repressed in females from *TP5* sn^w.

More than a year after obtaining the results in Table 2, we used RT-PCR to assess the level of transposase-encoding mRNA in females that might show the maternal effect. In this experiment, RNA was isolated from six replicate samples of each of three female genotypes: (1) *TP5* sn^w/$y\ sn^+$; CP/+ and (2) $y\ sn^w$/$y\ sn^+$; CP/+, both derived from a cross

between *TP5* sn^w/$y\ sn^w$ females and $y\ sn^+$; CP males and (3) $y\ sn^w$/$y\ sn^+$; CP/+, which came from a cross between $+sn^w$/$y\ sn^w$ females and $y\ sn^+$; CP males. This last genotype served as a control. The polyadenylated RNA in each sample was reverse transcribed into cDNA and then PCR was performed to amplify specific cDNAs. No consistent differences were seen among the three types of samples in amplifications with primers for the *aubergine* gene (Fig. 2, panel A). However, amplification with primers specific for germ-line *CP* cDNA revealed that the samples from the *TP5* sn^w/$y\ sn^+$; CP/+ females consistently had less product than the samples from the control genotype (Fig. 2, panel B). These results indicate that germ-line *CP* mRNA is diminished by the combined maternal and zygotic effects of the *TP5* element – a result that accords with the findings in Fig. 1. There was, however, no consistent evidence for a reduction in germ-line *CP* mRNA by a strictly maternal effect of the *TP5* element. Only two of the six samples from the $y\ sn^w$/$y\ sn^+$; CP/+ females that had *TP5* sn^w/$y\ sn^w$ mothers showed less RT-PCR product than the corresponding control samples.

In connection with this molecular analysis, we also performed genetic tests for repression of GD in females and sn^w mutability in males and females

Table 3. *Repression of P strain-induced GD and CP-induced* sn[w] *mutability in the offspring of* TP5 sn[w]/y sn[w] *females that had homozygous* TP5 sn[w] *mothers*

		GD			sn[w] mutability in males			sn[w] mutability in females		
Stock	TP5[a]	No. of vials	No. of flies	%GD $\pm$ SE[b]	No. of vials	No. of flies	Rate $\pm$ SE[c]	No. of vials	No. of flies	Rate $\pm$ SE[d]
w sn[w]	−[e]	25	256	100	31	892	0·469 $\pm$ 0·020	21	489	0·155 $\pm$ 0·025
	−[f]	25	242	100	32	1044	0·497 $\pm$ 0·019	28	738	0·137 $\pm$ 0·015
TP5 sn[w]	+	26	277	23·7 $\pm$ 5·6*	31	794	0·212 $\pm$ 0·041*	26	800	0·068 $\pm$ 0·016*
	−	26	252	28·6 $\pm$ 5·5*	31	661	0·409 $\pm$ 0·021	30	811	0·091 $\pm$ 0·016

In these experiments, stock females were crossed to *y sn[w]* males at 25 °C and their *TP sn[w]/y sn[w]* F$_1$ daughters were then either crossed to Harwich *y w* males at 29 °C to induce GD or to *y sn[+]*; *CP* males at 25 °C to obtain flies for the *sn[w]* mutability tests and RT-PCR assays (see Fig. 2). For the *sn[w]* mutability tests, F$_2$ *TP sn[w]* (grey) and *y sn[w]* (yellow) males were individually crossed to *C(1)DX* females and *TP sn[w]/y sn[+]* (grey) and *y sn[w]/y sn[+]* (yellow) females were individually crossed to *sn[3]* males to produce the F$_3$ flies that were scored. Significant repression of GD or *sn[w]* mutability relative to the corresponding *w sn[w]* control is indicated by an asterisk.
[a] *TP* present (+) or absent (−) in F$_2$ flies.
[b] Unweighted average percentage GD $\pm$ standard error.
[c] Unweighted average frequency of sn[(+)] and sn[e] sons among all sons $\pm$ standard error.
[d] Unweighted average frequency of sn[(e)] offspring among sn[w] and sn[e] offspring $\pm$ standard error.
[e] F$_2$ flies with grey bodies.
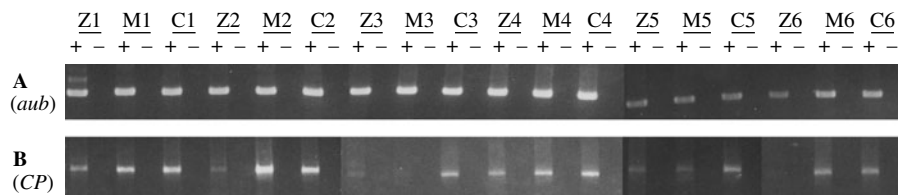[f] F$_2$ flies with yellow bodies.

Fig. 2. RT-PCR analysis of germline mRNA from the daughters of crosses between *TP5 sn[w]/y sn[w]* or *+sn[w]/y sn[w]* females and *y sn[+]*; *CP* males. RNA was independently obtained from six samples of each of three different genotypes: *TP5 sn[w]/y sn[+]*; *CP/+*, reflecting the zygotic (Z) and maternal effects of the *TP5* element from the strong repressing *TP5 sn[w]* strain; *y sn[w]/y sn[+]*; *CP/+*, reflecting the strictly maternal (M) effects of this element; and *y sn[w]/y sn[+]*; *CP/+*, a control lacking any effect of the *TP5* element; see text for details. A plus denotes where a sample has been reverse transcribed, and a minus denotes where it has not. (A) Amplification over 25 cycles using primers Aub-d and Aub-u to detect an 848-bp product from *aubergine* mRNA. (B) Amplification over 30 cycles using primers P0/1-d and P△2/3-u to detect a 1495-bp product from *CP* germ-line mRNA.

(Table 3). All the tests provided evidence for significant repression by a combination of maternal and zygotic effects. However, only the GD tests showed significant repression by a maternal effect alone. The *sn[w]* mutability tests hinted at such an effect, but the reductions in mutability seen in males and females were not statistically significant.

4. Discussion

Extensive data now indicate that the P cytotype is created by *P* elements that have inserted in the TAS at the left end of the X chromosome. Several different *P* element insertions in this locus have been identified and analysed.

For example, *TP5* and *TP6* are inserted in the same orientation at the same nucleotide position within one of the repeat sequences in the TAS; however, these elements differ in size – *TP6* is 101 bp longer, and in sequence – *TP5* is missing base pairs 438 through 1523, whereas *TP6* is missing base pairs 833 through 1816 from the 2907 base pairs of the canonical *P* element (Stuart *et al.*, 2002). Strains homozygous for either of these telomeric *P* elements vary in their ability to repress hybrid dysgenesis. Among the four strains studied here, one *TP5* strain was the strongest repressor and the other *TP5* strain was the weakest. The two *TP6* strains lay between these extremes. The consistent ranking of these four strains across three different repression assays argues that variation in repression ability cannot simply be due to structural differences between the *TP5* and *TP6* elements. Rather, it must involve some other factor such as the overall structure of the telomere into which each element is inserted.

Drosophila telomeres consist of two distinct regions: the proximal TAS (Karpen & Spradling, 1992), comprising a variable number of repeating units,

which are themselves variable in nucleotide sequence, and a distal array of retrotransposons (Mason & Biessmann, 1995). The retrotransposon array is capped at its end – the chromosome's end – by a protein complex that includes heterochromatin protein 1 (HP1), the product of the *Su(var)205* gene (Fanti *et al.*, 1998). Over time, the retrotransposon array changes. Sequences are lost because of incomplete DNA replication at the end of the chromosome, and sequences are gained when retrotransposons are added to the array. Because of these changes – and possibly others within the TAS, the telomeres in independently maintained stocks are expected to differ from one another, and such differences are known to have functional correlates. Thus, for example, extension of the retrotransposon array can enhance the expression of a transgene inserted within the TAS (Golubovsky *et al.*, 2001). Evolving telomere structure may therefore explain why particular *TP5* and *TP6* strains differ in their ability to repress hybrid dysgenesis – for example, why Stuart *et al.* (2002) found that in a pair of *TP5 sn^w* and *TP6 sn^w* strains, the *TP5 sn^w* strain was the better repressor of *sn^w* mutability, whereas in a pair of *TP5 sn^+* and *TP6 sn^+* strains, the *TP6 sn^+* strain was the better repressor of GD.

To investigate the molecular correlates of these differences, we used RT-PCR to assess the relative abundance of germ-line *P* mRNAs in females derived from the strong and weak repressing *TP5* strains. Females from the weak repressing strain produced more *TP5* mRNA in the germ line than their counterparts from the strong repressing strain. The mechanism of repression therefore cannot involve a polypeptide encoded by the *TP5* mRNA. Jensen *et al.* (2008) reached this same conclusion on the basis of a different argument. Instead of repressing through polypeptide products, telomeric *P* elements are now thought to repress hybrid dysgenesis by generating non-coding piRNAs that are primarily antisense in orientation (Brennecke *et al.*, 2008). These molecules are presumably synthesized from antisense transcripts that originate from a promoter within the TAS downstream of the inserted *P* element. This downstream antisense promoter might have been impaired in the weak repressing *TP5′* strain, or the promoter of the *TP5* element in this strain might have been upregulated so that sense transcripts are formed in place of antisense transcripts. Such upregulation might be due to the influence of enhancers located in the distal retrotransposon array. Another possibility is that the retrotransposon array in the weak repressing strain initiates transcripts that read sense-ward through the *TP5* element. How well a particular telomeric *P* element represses hybrid dysgenesis may therefore depend on the predominant direction of transcription through that element – determining, ultimately, how much antisense piRNA it can make.

We also examined the levels of transposase-encoding *P* mRNA in females derived from the strong and weak repressing *TP5* strains. The females from the strong repressing strain had less transposase mRNA than the females from the weak repressing strain. This finding suggests that the strength of repression depends on the ability of the piRNAs generated from a telomeric *P* element to destroy transposase mRNA. Strong repression evidently involves a more vigorous attack by these piRNAs than weak repression.

The *Drosophila* genome contains many loci that produce piRNAs (Brennecke *et al.*, 2007). So far, only one of these loci – in the TAS at the left end of the X chromosome – has been implicated in *P*-element regulation. A *P* insertion into this locus allows it to synthesize piRNAs with *P*-element specificity. In essence, the insertion 'marks' the piRNA-producing system for study. Genetic analyses of *P* regulation by these types of insertions may therefore provide information about the general features of this system. Thus, for example, the ability of a telomeric *P* element to regulate the *P* family is established in the female germ-line (Niemi *et al.*, 2004), and once established, this ability can be transmitted by females to their offspring independently of the telomeric *P* element itself. Patroclinously transmitted telomeric *P* elements utterly lose their regulatory ability; however, a telomeric *P* element inherited from a male can reacquire regulatory ability after passing through a female germ line, and this reacquisition is enhanced by the maternal and zygotic effects of another telomeric *P* element – a phenomenon called presetting (Niemi *et al.*, 2004), or the pre-P cytotype (Ronsseray *et al.*, 1993).

In terms of the piRNA model of *P*-element regulation, these genetic findings imply (1) that piRNAs are generated in the female germ line, (2) that they are transmitted to the next generation through the egg cytoplasm and (3) that they play a key role in defending against the *P* elements that are transmitted paternally in dysgenic crosses. In regard to this last point, Brennecke *et al.* (2007, 2008) have proposed that antisense piRNAs from a mother's telomeric *P* element interact with sense mRNAs encoded by paternally transmitted *P* elements to create a class of sense piRNAs, which then feed back to stimulate the synthesis of more antisense piRNAs, thereby amplifying the regulatory system that represses hybrid dysgenesis. This 'ping-pong' mechanism requires that both sense and antisense piRNA sources be present in the genome of the offspring. When the offspring do not inherit the mother's telomeric *P*, the maternally transmitted piRNAs can provide only a limited defence against dysgenesis. However, if some of the paternally transmitted *P* elements are able to generate antisense piRNAs – in effect, replacing the telomeric

P element that was not inherited maternally, this limited defence can be augmented by ping-pong cycling, starting with maternally inherited antisense piRNAs. This strengthening of *P* regulation is possible when the paternally transmitted *P* elements come from a P strain such as Harwich, which, because it has the P cytotype, must contain some *P* elements that are able to generate antisense piRNAs. However, when the only paternally inherited *P* element is in the *CP* transgene, it is difficult to see how more antisense piRNAs could be produced. The absence of a new source of antisense piRNAs may explain why the repression of sn^w mutability in the $y\ sn^w/sn^+$; $CP/+$ daughters of $TP5\ sn^w/y\ sn^w$ mothers and $y\ sn^+$; CP fathers is modest and not statistically significant, and it may also explain our inability to detect a consistent reduction in the amount of transposase-encoding *P* mRNA in these daughters.

Males that inherit piRNAs but not a telomeric *P* element from their mothers do not repress dysgenesis, as assayed by sn^w mutability, even when they inherit a host of *P* elements from Harwich fathers. The numerous cell divisions that occur in the germ lines of these males might dilute the maternally inherited piRNAs down to a point at which they are ineffective in preventing *P* excisions from the sn^w allele. In addition, the ping-pong mechanism is apparently stymied in these males, possibly because they lack a locus capable of generating antisense piRNAs. In the Harwich genome, the antisense piRNA locus may reside on the X chromosome, which these males did not inherit. By contrast, males that inherit a telomeric *P* element maternally can become strong repressors of sn^w mutability. The telomeric piRNA locus therefore is able to function in males. However, this function clearly depends on the locus being inherited maternally because patroclinously transmitted telomeric *P* elements do not repress sn^w mutability at all. Perhaps the female germ line imprints the locus in some way, or perhaps maternally transmitted piRNAs are needed to allow its expression in the next generation. In connection with this latter hypothesis, it is known that in females a paternally inherited telomeric *P* element reacquires its repression ability more fully if the mothers of these females carried a telomeric *P* element, even when the females do not inherit the element itself (Niemi *et al.*, 2004; Josse *et al.*, 2007). Maternally inherited piRNAs might therefore be involved in triggering, stabilizing, or enhancing the expression of the telomeric piRNA locus, possibly through an epigenetic alteration of its chromatin.

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