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Cytotype Regulation by Telomeric *P* Elements in *Drosophila melanogaster*: Interactions With *P* Elements From M′ Strains

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ABSTRACT

P strains of Drosophila are distinguished from M strains by having *P* elements in their genomes and also by having the P cytotype, a maternally inherited condition that strongly represses *P*-element-induced hybrid dysgenesis. The P cytotype is associated with *P* elements inserted near the left telomere of the *X* chromosome. Repression by the telomeric *P* elements *TP5* and *TP6* is significantly enhanced when these elements are crossed into M′ strains, which, like P strains, carry *P* elements, but have little or no ability to repress dysgenesis. The telomeric and M′ *P* elements must coexist in females for this enhanced repression ability to develop. However, once established, it is transmitted maternally to the immediate offspring independently of the telomeric *P* elements themselves. Females that carry a telomeric *P* element but that do not carry M′ *P* elements may also transmit an ability to repress dysgenesis to their offspring independently of the telomeric *P* element. Cytotype regulation therefore involves a maternally transmissible product of telomeric *P* elements that can interact synergistically with products from paternally inherited M′ *P* elements. This synergism between *TP* and M′ *P* elements also appears to persist for at least one generation after the *TP* has been removed from the genotype.

HYBRID dysgenesis is a syndrome of abnormal traits that occurs in the offspring of crosses between certain kinds of *Drosophila melanogaster* strains (Kidwell *et al.* 1977; Bregliano and Kidwell 1983; Engels 1989). This syndrome is characterized by high mutation rates, the occurrence of chromosome rearrangements, and sterility. Different families of transposable elements are responsible for hybrid dysgenesis. However, most research on this phenomenon has focused on the *P* elements, which are cut-and-paste transposons whose movement is restricted to germline cells.

P-element movement is catalyzed by an 87-kDa polypeptide, the P transposase, which is encoded by structurally complete members of the *P*-element family (Karess and Rubin 1984; Rio *et al.* 1986); these elements are 2907 bp long. Many different types of incomplete *P* elements are also found in *D. melanogaster* genomes. Incomplete *P* elements cannot produce the transposase, but they can be mobilized by it as long as they have transposase target sequences in both their left and right ends (Rio 1990).

P-element movement is restricted to the germline because the introns present in the transposase gene are fully removed from *P* transcripts only in germline cells (Laski *et al.* 1986). In somatic cells, the last *P* intron remains in the *P* RNA and prevents the synthesis of the catalytically active transposase. In its place, a shorter polypeptide is produced. This 66-kDa polypeptide is also made in germline cells, where it partially represses *P*-element activity (Misra and Rio 1990; Gloor *et al.* 1993; Simmons *et al.* 2002a). Polypeptides encoded by some incomplete *P* elements—in particular, the protein product of a 1.2-kb *P* element called *KP*—also function as partial repressors of hybrid dysgenesis (Black *et al.* 1987; Andrews and Gloor 1995; Simmons *et al.* 2002b).

For 3 decades, *D. melanogaster* strains have been classified into two broad categories, M and P, according to whether or not they yield dysgenic hybrids when they are crossed (Kidwell *et al.* 1977). Crosses between M females and P males produce dysgenic hybrids, whereas the reciprocal crosses, P females × M males, usually do not and neither do crosses between two different M strains or between two different P strains. These observations imply that P strains possess an ability to induce hybrid dysgenesis when they contribute paternally to crosses with M strains and that they also possess an ability to repress hybrid dysgenesis when they contribute maternally in crosses to other P strains (or to themselves). P–M hybrid dysgenesis is most easily detected by noting the occurrence of sterility in females (Engels and Preston 1979; Kidwell and Novy 1979). This sterility is due to the failure of the germline tissues

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to develop. Females with this defect, called gonadal dysgenesis (GD), cannot produce eggs—a trait that can be readily scored in each individual examined.

The classification of *D. melanogaster* strains on the basis of the results of crosses roughly coincides with a classification based on the presence or absence of *P* elements in genomes—that is, P strains possess *P* elements and M strains lack them (Bingham *et al.* 1982). Furthermore, P strains possess a state called the P cytotype, which strongly represses *P*-element movement, and M strains have a complementary state called the M cytotype, which permits it (Engels 1979a, 1989). Genetic analyses have indicated that the ability to repress hybrid dysgenesis (*i.e.*, the P cytotype) depends on the presence of *P* elements in the genome (Engels 1979a; Kidwell 1981; Sved 1987). The *P*-element family is therefore autoregulated.

There are, however, many exceptions to the simple classification of strains as P or M. Some strains with *P* elements in their genomes do not induce hybrid dysgenesis, or induce it very weakly, when they contribute paternally in crosses to M strains; however, they do repress hybrid dysgenesis when they contribute maternally in crosses to P strains—that is, they have the P cytotype. These strains have therefore been considered to be versions of P strains that do not induce hybrid dysgenesis effectively. They have been termed Q strains (Simmons *et al.* 1980; Engels and Preston 1981; Kidwell 1981; Bingham *et al.* 1982). Other strains have *P* elements in their genomes but they do not repress hybrid dysgenesis effectively when they contribute maternally in crosses to P strains, and neither do they induce hybrid dysgenesis when they contribute paternally in crosses to M strains (Bingham *et al.* 1982). Because these strains behave somewhat like M strains, they have been termed M′ or pseudo-M (Kidwell 1985; Simmons and Bucholz 1985). Both Q and M′ types are prevalent in surveys of strains derived within the past few decades from natural populations; see, for example, Anxolabéhère *et al.* (1985).

The history of genetics is replete with examples in which exceptions to a rule have provided key insights into biological phenomena. In this article, we use the Q and M′ exceptions to the simple P–M dichotomy to investigate the nature of cytotype regulation. In previous work, single *P* elements with the ability to repress hybrid dysgenesis were isolated from the genomes of two Q strains, ν_6 and Mt. Carmel (Stuart *et al.* 2002). These elements are inserted in the *t*elomere-*a*ssociated *s*equences (TASs) at the left end of the *X* chromosome. A large body of work by Stéphane Ronsseray, Dominique Anxolabéhère, and colleagues has shown that strains carrying only *P* elements inserted in the *X*-linked TAS repress hybrid dysgenesis, sometimes strongly (Ronsseray *et al.* 1991, 1993, 1996, 1998; Marin *et al.* 2000). The telomeric *P* elements isolated from ν_6 and Mt. Carmel repress hybrid dysgenesis only when they are transmitted maternally in crosses (Simmons *et al.* 2004). Because maternal transmission is a key feature of the P cytotype, these (and other) telomeric *P* elements may play an important role in establishing this powerful system of *P*-element regulation. Two M′ strains, Sexi and Muller-5 Birmingham, have also been shown to repress hybrid dysgenesis, albeit weakly (Kidwell 1985; Simmons and Bucholz 1985; Simmons *et al.* 1987, 1990). These strains may have an incipient or latent version of the P cytotype, or they may have some other feature that enables them to repress *P*-element activity.

In this article, we report the effect of combining the isolated telomeric *P* elements (*TP*s) from ν_6 and Mt. Carmel with the plethora of *P* elements from the M′ strains Sexi and Muller-5 Birmingham. Our study was motivated by the work of Ronsseray *et al.* (1998), who discovered interactions between telomeric *P* elements, telomeric *P* transgenes, and *P* elements from different P strains. However, one important difference between our study and theirs is that none of the interacting strains, either TP or M′, in our experiments carried complete *P* elements. Thus, there was no possibility for the synthesis of either the P transposase or the 66-kDa repressor polypeptide.

We find that hybrid dysgenesis is repressed much more strongly by the TP–M′ combinations than by the *TP* or M′ *P* elements themselves—that is, telomeric *P* elements interact with other *P* elements to create the strong system of repression that we call the P cytotype. At a mechanistic level, these interactions might reflect physical contact between the *TP* and M′ *P* elements so that a repressive factor—perhaps an imprint of telomeric heterochromatin—is transferred from the telomere to *P* elements scattered throughout the genome, or they might reflect the interplay of molecules produced separately by the *TP* and M′ *P* elements. On this latter hypothesis, the *TP* and M′ *P* elements might encode different polypeptides that work together to repress *P*-element activity, or they might generate *P* RNAs that trigger and sustain an RNA interference (RNAi) response. The evidence that we report here and in the accompanying article in this issue (Simmons *et al.* 2007) is consistent with the latter idea.

MATERIALS AND METHODS

Drosophila stocks and husbandry: The stocks, genetic markers, and special chromosomes are described on the FlyBase website (http://flybase.bio.indiana.edu/), in Lindsley and Zimm (1992), and in other references cited in the text. The TP5 and TP6 stocks have *P* elements inserted in the TASs at the left end of the *X* chromosome. The *TP5* element, originally isolated from the ν_6 Q strain, is 1.8 kb long and the *TP6* element, originally isolated from the Mt. Carmel Q strain, is 1.9 kb long (Stuart *et al.* 2002). These are the only *P* elements present in these stocks. Sexi.4 and Sexi.7 (Rasmusson *et al.* 1990) are highly inbred stocks derived from the M′ strain Sexi (Kidwell 1985). Neither of these stocks

TABLE 1

Gonadal dysgenesis in the offspring of TP and M′ strains

Stock	*P* elements present	No. of vials	No. of ♀♀	% GD ± SE[a]	Strain type
w	None	25[b]	447	100 ± 0.0	M
w m f	None	30[c]	366	100 ± 0.0	M
Samarkand (w^+)	None	25[b]	499	97.2 ± 1.9	M
		25[c]	332	99.7 ± 0.3	
Sexi.4(w^+)	43[d]	21[b]	255	100 ± 0.0	M′
		30[c]	484	100 ± 0.0	
Sexi.7(w^+)	33[d]	21[b]	359	83.7 ± 4.8	M′
		30[c]	446	93.7 ± 2.4	
M5B#1 (w^a *B*)	57[e]	24[b]	421	85.8 ± 3.5	M
		25[c]	226	95.4 ± 1.4	
TP5 (*w*)	1[f]	20[b]	367	50.2 ± 9.1	TP
TP6 (*w m f*)	1[f]	21[c]	202	9.6 ± 2.5	TP
Harwich (*w*)	ND[g]	25[c]	251	0.3 ± 0.3	P

[a] Unweighted mean percentage of GD ± standard error.
[b] Data obtained in conjunction with tests for interactions between *TP5* and M′ strains (see Table 2).
[c] Data obtained in conjunction with tests for interactions between *TP6* and M′ strains (see Table 2).
[d] *P* elements localized in euchromatin in polytene chromosomes (from Rasmusson *et al.* 1990).
[e] *P* elements localized in euchromatin in polytene chromosomes (from Simmons *et al.* 1987).
[f] The sole *P* element in these stocks is located at the left telomere of the *X* chromosome.
[g] Not determined.

contains any complete *P* elements, although both do contain *KP* elements (Simmons *et al.* 1990). M5B#1 (Simmons *et al.* 1987) is a highly inbred stock derived from the M′ strain Muller-5 Birmingham (Bingham *et al.* 1982). Like Sexi.4 and Sexi.7, M5B#1 does not contain any complete *P* elements; however, unlike the Sexi stocks, it also does not carry *KP* elements (Simmons *et al.* 1990). The presence of *KP* elements in the Sexi.4 and Sexi.7 strains and their absence in the M5B#1 strain was confirmed by PCR with a *KP*-specific primer; see Rasmusson *et al.* (1993) for the procedures used to carry out this confirming PCR. The autosomes in M5B#1 are denoted simply as *Birm*. Stocks containing either the *C(1)DX, y f* or *C(1)DX, y w f* attached-*X* chromosomes and *Birm* autosomes were synthesized by backcrossing attached-*X* females to M5B#1 males for 11 generations. The males in these stocks carried an *M5* balancer *X* chromosome derived from the M5B#1 stock. *X* chromosomes from M or TP stocks were substituted for this chromosome by backcrossing M or TP males to attached-*X*; *Birm* females for six generations. Strains homozygous for these M or TP *X* chromosomes were synthesized by crossing M; *Birm* or TP; *Birm* males from the attached-*X* stocks to M5B#1 females to obtain daughters heterozygous for either the M or the TP *X* chromosome and the *M5* balancer chromosome. These daughters were then crossed to M; *Birm* or TP; *Birm* males to obtain homozygous and hemizygous flies, which were used to establish stocks. Experimental cultures were reared at 25° on a cornmeal–molasses–yeast medium unless stated otherwise.

Gonadal dysgenesis assay for *P*-element activity: Females were mass mated at 21° to males from the strong P strain Harwich (Kidwell *et al.* 1977), which is marked with a null mutation in the *X*-linked *white* gene. After 3 days, each mated female was transferred to a fresh culture, which was incubated at 29°, a temperature that brings out high frequencies of gonadal dysgenesis (Engels and Preston 1979). On day 11, the progeny from each culture were transferred to a holding vial, and 2 days later, the females among them were scored for the presence or absence of eggs. The procedure was to squash the females between two glass slides in the presence of diluted food coloring, which helps to visualize the eggs. Females without any eggs were scored as having GD; females with one or more eggs were scored as normal. When different genotypes segregated from a cross, they were scored separately. Ideally, 20 females representing each genotype were scored from each culture; however, the actual numbers often fell short of this goal. Schemes to produce females for the GD assay are described in the results.

Statistical analysis: Differences among experimental groups were assessed by performing *z*-tests. The standard errors for these tests were obtained by using variances calculated empirically from independent replicate cultures.

RESULTS

Synergistic repression of gonadal dysgenesis in the offspring of hybrids between TP and M′ strains: Table 1 presents data on the frequency of gonadal dysgenesis among females produced by crossing males from the P strain Harwich to females from different M, M′, and TP strains, and from Harwich itself. Among these strains, only Harwich produces the P transposase. The M strains do not possess *P* elements, and the M′ and TP strains carry only incomplete *P* elements incapable of producing the P transposase (Simmons *et al.* 1987, 1990; Stuart *et al.* 2002). As expected, virtually all the females from the crosses to the three M strains exhibited GD, whereas almost none of the females from the crosses to Harwich did. The result from the cross of Harwich males to Harwich females is typical of P strains, which repress GD almost completely. Less complete repression is seen when Harwich males are crossed to either M′ or TP females. Among the M′ strains, Sexi.4 did not repress GD, whereas Sexi.7 and M5B#1 appeared to repress it slightly (GD frequencies from 84 to 95%). Between the

TABLE 2

Gonadal dysgenesis in the offspring of reciprocal F_1 hybrids between TP and M′ strains

Stock	*TP* present in F_2 ♀♀	Cross I: TP ♀ × stock ♂ → F_1 ♀		Cross II: TP ♂ × stock ♀ → F_1 ♀	
		No. of F_2 ♀♀	%GD ± SE[a]	No. of F_2 ♀♀	% GD ± SE[a]
		Control crosses with TP5			
Samarkand (M)	+	304	94.7 ± 2.7	320[b]	96.8 ± 1.0
		257	97.2 ± 1.4	336[b]	99.8 ± 0.2
		Control crosses with TP6			
Samarkand (M)	+	328	64.8 ± 4.6	218	99.6 ± 0.4
		336	76.7 ± 4.3	246	100.0 ± 0.0
		M′ crosses with TP5			
Sexi.4 (M′)	+	270	34.5 ± 4.2	403	96.6 ± 1.6
		267	45.7 ± 5.2	371	97.4 ± 1.0
Sexi.7 (M′)	+	323	39.6 ± 4.9	376	69.5 ± 5.6
		314	44.3 ± 5.5	353	67.0 ± 4.3
M5B#1 (M′)	+	308	43.6 ± 4.7	356	85.6 ± 2.8
		256	44.9 ± 4.9	297	88.5 ± 2.6
		M′ crosses with TP6			
Sexi.4 (M′)	+	406	1.1 ± 0.5	374	76.1 ± 7.6
		411	2.1 ± 0.7	420	76.5 ± 7.6
Sexi.7 (M′)	+	337	3.8 ± 2.4	275	70.7 ± 6.6
		312	0.9 ± 0.5	274	70.9 ± 6.6
M5B#1 (M′)	+	297	0.0 ± 0.0	315	7.4 ± 2.9
		265	0.4 ± 0.4	288	13.2 ± 4.2
		M′ crosses with *w m f* (M strain)[c]			
Sexi.4 (M′)		468	100.0 ± 0.0	478	100.0 ± 0.0
Sexi.7 (M′)		449	100.0 ± 0.0	452	99.6 ± 0.3
M5B#1 (M′)		421	98.7 ± 0.6	479	99.8 ± 0.2

These data were obtained from two separate experiments, one involving crosses with the TP5 strain and the other involving crosses with the TP6 and *w m f* strains.

[a] Unweighted mean percentage of GD ± standard error.

[b] In these groups, 24 F_1 females were tested; in all other groups, 25 F_1 females were tested.

[c] The M strain *w m f* was substituted for the TP strains in crosses I and II, and the F_2 females were scored for GD without regard to eye color.

TP strains, TP5 repressed GD moderately (GD frequency 50%) and TP6 repressed it strongly (GD frequency 10%). Thus, the M′ and TP strains differ in their abilities to repress GD.

To see if the repression abilities of the TP and M′ strains could be combined, either additively or synergistically, we crossed each TP strain to each M′ strain and then mated the F_1 hybrid females to Harwich males and scored their daughters for GD. The F_1 females were produced by performing reciprocal crosses—TP female × M′ male (cross I) and TP male × M′ female (cross II)—and the two types of F_1 females were analyzed separately because they differ in the way in which the telomeric *P* element was inherited—maternally in cross I and paternally in cross II. Previous studies have indicated that the maternal transmission of telomeric *P* elements is important for the regulation of *P*-element activity (Marin *et al.* 2000; Stuart *et al.* 2002; Niemi *et al.* 2004; Simmons *et al.* 2004). As controls, we performed the same analysis using F_1 females produced by reciprocally crossing each of the TP strains to the M strain Samarkand (TP female × Samarkand male = cross I and TP male × Samarkand female = cross II). In all these crosses, the *X* chromosomes from the TP strains were marked with a null mutation of the *white* gene, which is tightly linked to the telomeric *P* element (~1.5% recombination), and the *X* chromosomes from the M and M′ strains were marked with either w^+ or w^a. Thus, we could distinguish which of the F_2 females from the testcrosses to Harwich males (which are hemizygous for a *w* null mutation) carried a telomeric *P* element (phenotypically w F_2 females) and which did not (phenotypically w^+ or w^a F_2 females). Table 2 summarizes the results of these experiments.

First, we consider the results with the controls, which involved hybrids between the TP strains and the M strain Samarkand ("Control crosses with TP," Table 2). For TP5, the daughters of these hybrids showed high frequencies of GD (>95%), regardless of which way the hybrids were produced. Thus, this strain does not

transmit its moderate ability to repress GD (see Table 1) through two generations, even when TP5 females are used in the first cross. For TP6, the daughters of the hybrids from cross II showed high frequencies of GD; however, those from cross I showed moderate frequencies. Thus, TP6 does transmit its ability to repress GD through two generations, providing that TP6 females are used in the first cross. Furthermore, both classes of the F_2 females derived from this cross had reduced frequencies of GD—65% for the females that carried the *TP6* element and 77% for those that did not; these values are significantly less than the 97–100% GD seen with tests of different M strains, but significantly greater than the 10% GD seen with tests of the TP6 stock itself (see Table 1). Thus, the *TP6/+* hybrids from cross I transmit the ability to repress GD to their daughters, and they do so independently of the inheritance of the *TP6* element itself. This observation provides evidence for the existence of a maternally transmitted component of cytotype regulation that is separable from the telomeric *P* element—that is, it shows that there is a *bona fide* "cytoplasmic" component in this system of regulation. Additional tests with the TP6 strain have confirmed this finding (M. Simmons and R. Wolff, unpublished results). A cytoplasmic component of repression has also been seen in the analysis of Lk-P(1A), a strain with two *P* elements inserted in the *X*-linked TAS (Ronsseray *et al.* 1993).

Next we consider the results from the hybrids between TP5 and the three different M′ strains ("M′ crosses with TP5," Table 2). Moderate frequencies of GD (35–46%) were seen in the offspring of the cross I hybrids, regardless of which M′ strain was involved and whether or not the *TP5* element was present in the F_2 genotype. These results indicate that GD is repressed significantly, and more or less uniformly, in the offspring of these hybrids and that the *TP5* element need not be present for this repression to occur. Note that the frequencies of GD seen here are significantly less than those seen in the cross I controls (95–97% GD) and that they are also significantly less than the frequencies seen in the tests with any of the M′ strains (>84% GD; see Table 1). The *TP5* and M′ *P* elements therefore seem to interact in the cross I hybrid females to create a regulatory state with an enhanced ability to repress GD in the next generation, even when the F_2 flies do not inherit the *TP5* element.

For the offspring of the TP5 × M′ hybrids from cross II, the GD frequencies were higher than those seen in the offspring from cross I, and they also varied among the M′ strains tested: 97% for Sexi.4, 87% for M5B#1, and 68% for Sexi.7. Furthermore, for each of the M′ strains, the F_2 flies that carried the *TP5* element had about the same GD frequency as the flies that did not. These results indicate that GD is repressed unevenly in the offspring of these hybrids—moderately with Sexi.7, weakly with M5B#1, and negligibly with Sexi.4—but that when repression does occur, the *TP5* element need not be present. The level of repression seen in the offspring of the Sexi.7 hybrids suggests an interaction between the *TP5* and Sexi.7 *P* elements. However, this interaction is evidently not as strong as the one that occurs in the corresponding cross I hybrids, whose offspring showed much less GD (40–45%). For Sexi.7, and for the two other M′ strains as well, maternal inheritance of the *TP5* element by the F_1 hybrid females leads to significantly stronger repression of GD in the next generation.

Now we consider the results from the various TP6 × M′ hybrids ("M′ crosses with TP6," Table 2). Very strong repression of GD was seen in the offspring of cross I (<4% GD), regardless of which M′ strain was involved. By contrast, the offspring of cross II showed either strong (7–13% GD) or moderate repression (71–76% GD), depending on the M′ strain. In all cases, however, the level of repression was about the same in the F_2 flies that inherited the *TP6* element as in those that did not. Thus, the *TP6* element need not be present in the F_2 for repression to occur.

The data from the TP6 × M′ hybrids indicate strong interactions between the *TP6* and M′ *P* elements. The control data from the TP6 × Samarkand cross I hybrids show that by itself the *TP6* element brings about moderate repression (65–77% GD). As already mentioned, the M′ strains are, at best, weak repressors of GD. However, the offspring of all the TP6 × M′ cross I hybrids showed very strong repression of GD, similar to a P strain such as Harwich. Thus, when inherited maternally, the *TP6* element interacts with *P* elements inherited paternally from the M′ strains to establish a highly effective regulatory state, which is then passed on to the next generation. Note that F_2 flies that do not inherit *TP6* repress GD as strongly as their *TP6*-carrying sibs. Thus, this regulatory state is transmitted independently of the *TP6* element itself. A less effective regulatory state is established through an interaction between the paternally contributed *TP6* element and the M′ *P* elements in the cross II F_1 females. However, once established, this state is also transmitted independently of the *TP6* element.

To assess whether or not the repression seen in the offspring of the TP × M′ hybrids is due to the simple addition of the repression ability of the *P* elements from an M′ strain to that of a telomeric *P* element, we need to know the repression ability of the M′ *P* elements by themselves. Furthermore, we need to know this ability in hybrid flies, not in the inbred M′ strains shown in Table 1. Accordingly, we tested reciprocal hybrids from crosses in which an M strain (*w m f*) replaced the TP strains in the mating schemes described above. These hybrids carry the same complement of *P* elements from the M′ strains as the TP × M′ hybrids, but they do not carry a telomeric *P* element. Thus, they permit an assessment of the ability of the M′ *P* elements to repress GD in a context comparable to that of the TP × M′ hybrids,

but in the absence of either *TP5* or *TP6*. The results of these tests are shown in Table 2, "M′ crosses with *w m f* (M strain)." In the hybrid context, the repression ability of the *P* elements from each of the M′ strains is nil. Thus, the repression seen in the offspring of the TP × M′ hybrids, which in all cases but one (TP5 × Sexi.4, cross II) is significantly greater than that seen in the offspring of the TP × M controls, cannot be explained by a simple additive model. Rather, it must be due to a synergistic interaction between the M′ *P* elements and the telomeric *P* elements.

Synergistic repression of gonadal dysgenesis requires the coexistence of the telomeric *P* elements and M′ *P* elements in females: The data showing that *TP* and M′ *P* elements interact synergistically to repress dysgenesis were obtained from crosses in which the *TP* and M′ *P* elements had coexisted in hybrid females. To see if this coexistence in females was necessary for synergistic repression to occur, we performed an analysis using TP–M′ stocks in which the *TP* element was present in males but not in females.

The stocks for this analysis were constructed by crossing the autosomes from the M5#1 strain—hereafter referred to as the *Birm* autosomes—into M strains with attached-*X* chromosomes. The *X* chromosome in the males of these stocks—the "free" *X* chromosome—was derived from either the TP5 or the TP6 strain or from an M strain marked with *w*. Females from all these stocks were tested for their ability to repress GD by crossing them to Harwich males. As controls, we tested females from similar attached-*X* stocks that did not carry the *Birm* autosomes. We also tested females from an attached-*X* stock that had been developed from the P strain π_2 (Engels 1979b). The results of all these tests are shown on the left side of Table 3.

None of the Birm attached-*X* stocks, including those with the *TP5* or *TP6* elements on the free *X* chromosome in males, repressed GD, and neither did any of the controls that had an M genetic background. In fact, the only attached-*X* stock that repressed GD was the one derived from the π_2 P strain, and it did so very effectively (2% GD). Thus, stocks that carry autosomal *P* elements from the M5B#1 strain in both sexes and an *X*-linked telomeric *P* element in males do not develop the synergistic interaction between these components that brings about repression of GD in their offspring.

To show that a synergistic interaction can develop if the *Birm* autosomes and an *X*-linked telomeric *P* element are brought together in females, we derived homozygous free *X* stocks from each of the Birm attached-*X* strains and then tested their abilities to repress GD by crossing females from these stocks to Harwich males. As shown by the data in the right columns of Table 3, the Birm stocks homozygous for an *X* chromosome that carried either *TP5* or *TP6* strongly repressed GD (for *TP5*, 1–18% GD and for *TP6*, 1–2% GD), whereas the Birm stocks homozygous for the *w* chromosome that came from an M strain completely failed to repress it. As controls, we also tested the *w*, TP5, TP6, and M5B#1 stocks that were used to construct the attached-*X* and homozygous *X* stocks analyzed in this experiment. The results of these controls are shown in Table 3. As expected, the *w* stock did not repress GD and the M5B#1 stock did so very slightly (95% GD). TP5 was a weak repressor (85% GD) and TP6 was a moderate repressor (32% GD). Clearly, the TP5 and TP6 root stocks were not nearly as effective in repressing GD as the TP5; Birm and TP6; Birm stocks. Thus, the telomeric and Birm *P* elements interact to repress GD, but only if they have coexisted in females.

Persistence of synergistic repression of gonadal dysgenesis after removing a telomeric *P* element from a TP; Birm stock: To see if the strong repression characteristic of the homozygous TP5; Birm and TP6; Birm stocks could persist in the absence of the telomeric *P* elements, we used a two-generation scheme to remove these elements from the stocks. TP; Birm females were crossed to +; Birm males, which carried a wild-type *X* chromosome devoid of *P* elements, to obtain *TP*/+; *Birm* F_1 females. One sample of these females was mated to Harwich males to test for repression of GD, and another sample was mated to *w*; Birm males to obtain *TP*/*w*; *Birm* and +/*w*; *Birm* F_2 females, which were then crossed to Harwich males to test for repression of GD. This crossing scheme allowed us to see if a diploid complement of *Birm* autosomes, once synergized by a telomeric *P* element, could retain the ability to repress GD after that element was removed from the genotype (in the +/*w*; *Birm* F_2 females).

The results of the tests with the F_1 hybrid females from four different TP; Birm stocks indicated that GD was repressed strongly (<2% GD) in their daughters regardless of whether or not the daughters inherited a telomeric *P* element (supplemental Table S1 at http://www.genetic.org/supplemental/). The strong synergism between the *TP* and M′ *P* elements is therefore maintained in these F_1 females, which have a diploid complement of *Birm* autosomes but are heterozygous for the *TP*.

The more complex results of the tests with the two types of F_2 females—those carrying and those not carrying a *TP*—are summarized in Table 4. For the F_2 females that carried a *TP*, we could not determine which of their daughters inherited the *TP*. However, as with the data from the F_1 females, the presence or absence of a *TP* did not seem to matter. GD was repressed strongly in the daughters of these F_2 females: 7% GD when *TP5* was segregating and <1% GD when *TP6* was segregating. Thus, the strong repression seen with the TP; Birm stocks was maintained in these F_2 females that carried a *TP*.

The F_2 females that did not carry a *TP* produced different results. These females had a diploid complement of *Birm* autosomes, but had not inherited a *TP*

TABLE 3

Gonadal dysgenesis in the offspring of TP; Birm stocks

Stock	Replicate[a]	Stocks with attached-*X* ♀♀			Stocks with homozygous *X* ♀♀		
		No. of vials	No. of ♀♀	% GD ± SE[b]	No. of vials	No. of ♀♀	% GD ± SE[b]
				Stocks with *Birm* autosomes[c]			
w	1a	25	385	100.0 ± 0.0	23	438	99.1 ± 0.4
	1b	23	267	100.0 ± 0.0	30	519	98.5 ± 0.6
	2a	9	41	100.0 ± 0.0	30	556	97.2 ± 0.9
	2b	25	293	100.0 ± 0.0	19	360	100.0 ± 0.0
TP5	1a	25	367	100.0 ± 0.0	30	584	17.1 ± 4.5
	1b	25	355	100.0 ± 0.0	26	473	18.1 ± 3.8
	2a	25	284	100.0 ± 0.0	27	482	0.7 ± 0.3
	2b	25	453	100.0 ± 0.0	26	384	1.9 ± 0.8
TP6	1a	25	424	100.0 ± 0.0	11	198	0.9 ± 0.9
	2a	25	281	99.6 ± 0.4	36	416	1.2 ± 0.8
	2b	23	261	99.6 ± 0.4	37	507	2.3 ± 0.9
				Control attached-*X* stocks[d]			
w	1	24	373	100.0 ± 0.0			
	2	24	328	100.0 ± 0.0			
TP5	1	24	460	100.0 ± 0.0			
	2	25	295	99.7 ± 0.2			
TP6	1	24	411	100.0 ± 0.0			
	2	25	398	99.5 ± 0.4			
π_2	1	22	328	2.1 ± 1.1			
				Control free-*X* stocks			
w					30	600	100.0 ± 0.0
TP5					32	596	85.4 ± 3.4
TP6					24	262	31.6 ± 6.0
M5B#1					24	277	94.8 ± 2.3

[a] Among the attached-*X* stocks, replicates 1, 1a, and 1b carried *C(1)DX, y f* and replicates 2, 2a, and 2b carried *C(1)DX, y w f*.

[b] Unweighted mean percentage of GD ± standard error (SE). Note that in the tests of females with attached-*X* chromosomes, GD is induced by autosomes and a *Y* chromosome derived from the Harwich P strain whereas, in the tests of females with homozygous *X* chromosomes, it is induced by autosomes and an *X* chromosome from this strain.

[c] Stocks with attached-*X* chromosomes and *Birm* autosomes were created by repeatedly backcrossing males that carried a particular free *X* chromosome to attached-*X* females that carried the *Birm* autosomes. Homozygous *X* stocks were created by crossing males from each of the attached-*X* stocks to M5B#1 females and then using the balancing properties of the *M5* chromosome to make the free *X* chromosome homozygous.

[d] The π_2 attached-*X* stock was derived from a P strain. It contains numerous *P* elements and carries the double *P*-element insertion mutation sn^{w} on its free *X* chromosome (Engels 1979b). The only *P* elements present in the other control attached-*X* stocks were *TP5* or *TP6*.

from their *TP*/+; *Birm* F_1 mothers. When testcrossed to Harwich males, they yielded daughters that had moderate-to-high frequencies of GD. The three moderate frequencies (64, 77, and 82% GD) suggested that the *Birm P* elements, once synergized by a *TP*, could retain some ability to repress GD even after the *TP* had been removed from the genotype. As a control for the intrinsic repression ability of the *Birm P* elements, we concurrently testcrossed *w*; *Birm* females from the stock used in this experiment to Harwich males and scored their daughters for GD. Among 282 daughters from 25 cultures, the frequency of GD was 98.9 ± 0.5%—significantly higher than the moderate frequencies reported in Table 4. Thus, the moderate repression seen with the F_2 females that lacked a telomeric *P* element appears to reflect a synergizing effect of the *TP* on the *Birm P* elements in previous generations and not an intrinsic effect of the *Birm P* elements themselves. This conclusion should, however, be accepted cautiously because, in a test done 8 months earlier, the +; Birm stock that was used in these experiments yielded 82.3% GD when crossed to Harwich males—a result statistically indistinguishable from two of the moderate GD frequencies in Table 4. If this previous result is used as a reference value for the data in Table 4, then only one of the TP; Birm stocks provided firm evidence for the persistence of synergism between the *TP* and *Birm P* elements after the *TP* had been removed from the stock.

DISCUSSION

Collectively, the data from the TP × M′ hybrids indicate that repression of GD by the telomeric *P* elements is strengthened when these elements are combined

TABLE 4

Gonadal dysgenesis in the grand offspring of F_1 hybrids between TP; Birm stocks and a +; Birm stock

	TP present in F_2 ♀♀			*TP* absent in F_2 ♀♀		
Stock[a]	No. of vials	No. of ♀♀	% GD + SE[b]	No. of vials	No. of ♀♀	% GD + SE[b]
TP5; Birm (2a)	25	452	6.7 ± 1.9	24	430	81.9 ± 4.9
TP5; Birm (2b)	25	465	6.6 ± 2.0	25	457	77.2 ± 5.6
TP6; Birm (2a)	25	472	0.4 ± 0.3	25	431	63.9 ± 5.7
TP6; Birm (2b)	25	432	0.6 ± 0.4	24	403	95.7 ± 1.5

The *TP* (*w*)/+; Birm F_1 hybrids were crossed to males from a *w*; Birm stock (2a in Table 3) and their *TP* (*w*)/*w*; Birm (phenotypically white, *TP* present) and +/*w*; Birm (red, *TP* absent) F_2 daughters were then crossed to Harwich males to obtain F_3 females, which were scored for GD regardless of eye color.

[a] Replicate stocks carrying the same *TP* are distinguished by the identity tags in parentheses; these tags correspond to the ones given in Table 3.

[b] Unweighted mean percentage GD ± standard error.

with incomplete *P* elements from an M′ strain—and sometimes dramatically so. The strongest repression was seen when *TP6* was combined in hybrids with M′ *P* elements and when the *TP6* element in these hybrids had been inherited maternally; in these cases, the repression was essentially complete. When the *TP6* element had been inherited paternally, weaker repression was seen. *P* elements from the M′ strains also strengthened the ability of *TP5* to repress GD, especially when the *TP5* element had been inherited maternally.

Experiments with attached-*X* strains indicated that telomeric *P* elements and M′ *P* elements need to coexist in females for synergistic repression of GD to occur. This observation is consistent with previous findings that the P cytotype is established in females but not in males and that patroclinous transmission of a telomeric *P* element abolishes its ability to repress hybrid dysgenesis (Niemi *et al.* 2004). Stocks that carried M′ *P* elements on their autosomes and that were homozygous for an *X*-linked telomeric *P* element proved to be strong repressors of GD—stronger if they carried *TP6* rather than *TP5*. Genetic analysis of these stocks suggested that the ability to repress GD could persist, albeit much diminished, even after the telomeric *P* element was removed from the genotype.

Repression of GD in the offspring of TP–M′ hybrids was more effective when the TP strain contributed maternally to the hybrids. This observation underscores the importance of a maternal contribution from the TP strain seen in previous studies that used a different assay (Stuart *et al.* 2002; Simmons *et al.* 2004). However, unlike the previous studies, the results reported here show that flies need not actually *inherit* a telomeric *P* element from their hybrid mothers to repress GD. This phenomenon was even seen in the offspring of the TP6 × M hybrids, in which no M′ *P* elements were present, and was noted for another strain, Lk-P(1A), in one of the first genetic analyses of cytotype regulation by telomeric *P* elements (Ronsseray *et al.* 1993). Thus, this system of repression involves a factor that can be transmitted through the egg independently of the telomeric *P* element itself. This "cytoplasmic" factor, which Ronsseray *et al.* (1993) called the "pre-P cytotype," is likely to be a product of the telomeric *P* element—either an RNA or a protein.

Some repression was also observed in the offspring of hybrids from crosses between TP males and M′ females. In one case (the offspring of hybrids from TP6 males and M5B#1 females), this repression was strong (only 7–13% GD). Thus, maternal inheritance of a telomeric *P* element by TP–M′ hybrids is not an absolute prerequisite for repression to occur in the offspring. Evidently, a paternally inherited telomeric *P* element can interact with maternally inherited M′ *P* elements to establish a state that will repress GD in the next generation. The coexistence of these elements in hybrid females is therefore sufficient to initiate the development of regulatory ability; that is, it appears to initiate the development of the P cytotype.

All three M′ strains tested provided evidence of interactions with the telomeric *P* elements. Among the offspring of the cross I hybrids (TP female × M′ male), the interactions were equivalently strong for all three M′ strains, although the actual level of repression was determined by whichever TP strain was involved: 35–45% GD for the offspring of the TP5 hybrids and <4% GD for the offspring of the TP6 hybrids. Among the offspring of the cross II hybrids (TP male × M′ female), Sexi.7 was the best interactor with TP5, and M5B#1 was the best interactor with TP6. These observations indicate that as long as the TP–M′ hybrids inherit a telomeric *P* element maternally, the attributes of the M′ *P* elements—their number, structure, and genomic positions—do not seem to matter. However, when the sexes are reversed and the telomeric *P* element is inherited paternally by the hybrids, these attributes may make a difference.

One possibility is that a telomeric *P* element might be present in a particular M′ strain, and the maternal inheritance of this *P* element might facilitate the establishment of the P cytotype. Among the three M′ strains

tested, only Sexi.4 has *P* elements in telomeric regions (1A on the *X* chromosome and 100F on chromosome *3*R). This information comes from the *in situ* hybridizations of *P* probes to larval salivary glands that were used to localize *P* elements in the M′ chromosomes; see Rasmusson *et al.* (1990). However, this strain was the poorest interactor in cross II with either TP5 or TP6. Another possibility is that the number of *P* elements in the M′ strains—ascertained by counting sites on polytene chromosomes that had been labeled by *P*-element probes—accounts for the differences seen with the cross II hybrids. However, for the TP5 hybrids, those with the fewest M′ *P* elements (from Sexi.7) were the best repressors of GD in the next generation, whereas for the TP6 hybrids, those with the most M′ *P* elements (from M5B#1) were the best repressors. Another hypothesis is that the strength of the interaction in the cross II hybrids depends on the presence of *P* elements that encode repressor polypeptides. For instance, both Sexi.4 and Sexi.7 contain *KP* elements whereas M5B#1 does not. However, this hypothesis does not explain why Sexi.4 and Sexi.7 are much less effective interactors than M5B#1 in crosses with the TP6 strain. Thus, from these considerations, it is not clear what attributes of the M′ *P* elements are important for interactions with the telomeric *P* elements in the cross II hybrids. It may be that these interactions depend on the genomic locations of the M′ *P* elements or on their levels of expression.

Of the two telomeric *P* elements used in this study, *TP6* has consistently been the better repressor of GD. By contrast, *TP5* has been the better repressor of *P*-element excisions from the *X*-linked *singed* gene (Stuart *et al.* 2002; Simmons *et al.* 2004; Niemi *et al.* 2004). These differences are somewhat surprising because the two elements are similar in size and are inserted in the same position in one of the TAS repeats at the left end of the *X* chromosome. The different properties of these elements may therefore be a consequence of their particular DNA sequences or of a peculiarity of the particular telomere in which they are inserted.

What do the interactions between telomeric and M′ *P* elements imply for a mechanistic understanding of the P cytotype? One possibility is that these elements interact physically; that is, telomeric *P* elements pair ectopically with other *P* elements, and this pairing somehow enhances the development of the P cytotype, perhaps by transferring an imprint of telomeric heterochromatin to other *P* elements. This idea was suggested by Ronsseray *et al.* (1998), who observed strong interactions between telomeric *P* elements and *P* elements from different P strains. They also found strong interactions between telomeric *P* elements and *P* transgenes inserted in TAS on chromosomes *X* and *3*R. From these results and from evidence that the silencing of *P* transgenes by telomeric *P* elements is homology dependent (Roche and Rio 1998; Marin *et al.* 2000), Stuart *et al.* (2002) hypothesized that physical interactions between the telomeric *P* elements *TP5* and *TP6* and other *P* elements might play an important role in establishing and maintaining the P cytotype.

There is, however, a more likely possibility. The synergistic repression seen in TP–M′ combinations might be due to interactions between the products of telomeric and M′ *P* elements. These elements might, for example, encode different polypeptides that assemble into a complex that represses *P*-element movement; however, there is little indication that the intrinsic repression abilities of *TP5* and *TP6* are due to repressor polypeptides (Stuart *et al.* 2002; P. Jensen, J. Stuart, M. Goodpaster, K. Newman, J. Goodman and M. Simmons, unpublished results). A more plausible scenario is that the strong repression created by combining the *TP* and M′ *P* elements is due to an RNA interference mechanism triggered by transcripts from the telomeric *P* element and amplified by transcripts from the M′ *P* elements. This mechanism could repress hybrid dysgenesis by targeting the RNA interference machinery to *P*-element mRNA, thereby preventing the synthesis of the P transposase, which is needed to mobilize *P* elements in the genome. The finding that repression of hybrid dysgenesis by telomeric *P* elements is profoundly disrupted by mutations in a gene involved in RNA interference gives credence to this hypothesis (Reiss *et al.* 2004; Simmons *et al.* 2007, accompanying article in this issue). Furthermore, Brennecke *et al.* (2007) have implicated the TAS near the left end of the *X* chromosome in the production of small RNAs associated with the proteins involved in the RNAi response. They have also outlined a process whereby this response can be amplified by RNAs from different sources and have suggested how the amplified response might persist after the triggering agent—in this case, a *P* element inserted in the TAS—is removed from the genotype. Some of our data (Table 4) suggest this sort of persistence. The synergistic repression of hybrid dysgenesis by *TP* and M′ *P* elements may therefore be one example of how an RNAi response is amplified to regulate the activity of a family of transposable elements.

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