The Unusual Spectrum of Mutations Induced by Hybrid Dysgenesis at the *Triplo-lethal* Locus of *Drosophila melanogaster*

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ABSTRACT

The *Triplo-lethal* locus (*Tpl*) is unique in its dosage sensitivity; no other locus in Drosophila has been identified that is lethal when present in three doses. *Tpl* is also haplo-lethal, and its function is still a mystery. Previous workers have found it nearly impossible to mutationally inactivate *Tpl* other than by completely deleting the chromosomal region in which *Tpl* resides (83DE). We have utilized P-M hybrid dysgenesis in an effort to obtain new mutations of *Tpl*. We recovered 19 new duplications of *Tpl*, 15 hypomorphic mutations of *Tpl* (a previously rare class of mutation), and no null mutations. Surprisingly, 14 of the 15 hypomorphic alleles have no detectable *P* element sequences at the locus. The difficulty in recovering null mutations in *Tpl* suggests that it may be a complex locus, perhaps consisting of several genes with redundant functions. The relative ease with which we recovered hypomorphic alleles is in sharp contrast to previous attempts by others to mutagenize *Tpl*. A higher mutation rate with hybrid dysgenesis than with radiation or chemicals also suggests a peculiar genetic organization for the locus.

IN *Drosophila melanogaster* there is only one locus that is triplo-lethal; this locus, *Tpl*, is also haplo-lethal and is located on the third chromosome at cytological location 83D4,5-E1,2 (LINDSLEY *et al.* 1972; DENELL 1976; KEPPY and DENELL 1979). Individuals carrying three doses or one dose of *Tpl* die either as late embryos or early first instar larvae (DENELL 1976). No morphological abnormalities have been reported in the dying embryos. Extensive mutational analysis has not revealed the function of *Tpl*; however, the results of mutagenesis have suggested that *Tpl* may be complex or unusual (KEPPY and DENELL 1979; ROEHRDANZ and LUCCHESI 1980). Selection of new mutations at *Tpl* depends on the existence of balanced stocks carrying a duplication of *Tpl* on one homolog and a deficiency on another. When these stocks are crossed to wild-type flies, all progeny will die due to aneuploidy for *Tpl*. If the wild-type parents are mutagenized, then exceptional progeny may result; these should carry either a new duplication or a new deficiency of *Tpl*. In general when ionizing radiation is used as a mutagen, chromosomal aberrations are expected, and in the case of *Tpl* this is what is observed. KEPPY and DENELL (1979) used γ-rays as a mutagen and recovered several deficiencies of *Tpl* at a mutation rate of approximately 1.8×10^{-4}. No inversion or translocation breakpoints that inactivate *Tpl* were recovered. In the same study they also used the chemical mutagens ethyl methanesulfonate (EMS) and formaldehyde, and recovered inactivations of *Tpl* at the surprisingly low rate of 2.3×10^{-5}. Of the inactivations recovered following EMS mutagenesis the majority were cytologically visible deficiencies. KEPPY and DENELL (1979) interpreted these results to indicate that single base-pair substitutions that inactivate *Tpl* function are very rare, or even nonexistent.

ROEHRDANZ and LUCCHESI (1980) also carried out EMS mutagenesis of *Tpl* and found a very low mutation rate (5×10^{-5}). Of the seven mutations they recovered, four behaved as complete inactivations of *Tpl* and were apparently large deficiencies, supporting KEPPY and DENELL's (1979) suggestion that single base-pair changes that inactivate *Tpl* are rare or nonexistent. The remaining three mutations recovered by ROEHRDANZ and LUCCHESI (1980) were of a new class. These were recovered in the selection because they rescued flies carrying the duplication bearing chromosome, and they are homozygous lethal, but unlike deletions they are viable when heterozygous with a wild-type chromosome. These three alleles (Tpl^{10}, Tpl^{17} and Tpl^{38}) behave, therefore, as hypomorphs. They are cytologically normal and do not complement one another for the recessive lethality (ROEHRDANZ and LUCCHESI 1980). Although they are cytologically normal, they do not behave as complete inactivations of the locus, again suggesting that simple lesions do not readily inactivate *Tpl* function. In addition, these three hypomorphic alleles are site-specific hotspots for recombination (DORER and CHRISTENSEN 1989), which led us to suggest that they were

due to transposon insertions rather than single base-pair changes.

We have carried out a selection for new mutations of *Tpl* using hybrid dysgenesis. When mutations are isolated following hybrid dysgenesis, it is usually found that they are due to insertions of the *P* transposable element (ENGELS 1989). We have obtained a large number of new duplications of *Tpl*, as well as 15 new hypomorphic alleles. Surprisingly, we did not recover any null mutations of *Tpl*. This again suggests that it is not a simple matter to completely inactivate *Tpl* function without removing the entire locus from the genome. Also surprisingly, we found that the hypomorphic alleles induced by hybrid dysgenesis do not appear to be due to insertions of the *P* element as assayed by *in situ* hybridization to polytene chromosomes. The implications of these results for the genetic structure of the *Tpl* locus are discussed.

MATERIALS AND METHODS

Drosophila media, stocks and culture conditions: Stocks were maintained and fed as described in DORER and CHRISTENSEN (1989). The *Tpl* duplication over deficiency stock used in this study was *C(1)M3 y² bb/Y/Y^S X.Y^L, In(1)EN y;;Dp(3;3)21l73 p^p/Df(3R)18i77 p^p*. The origin of this stock was described in DORER and CHRISTENSEN (1989) and KEPPY and DENELL (1979). The *Dp(3;3)21l73* is also referred to as *Dp(3;3)E6*, *Dp(3;3)83D-E*, and most recently as *Dp(3;3)Tpl21* (LINDSLEY and ZIMM 1987). In this work we will abbreviate *Dp(3;3)21l73* as *Dp(Tpl)* and *Df(3R)18i77* as *Df(Tpl)*. Alterations of *Tpl* produced in this study will be referred to specifically by name, or generically by the superscript '*hd*' for hybrid dysgenesis. Other genetic markers are described in LINDSLEY and GRELL (1968) or DORER and CHRISTENSEN (1989), except the π_2 stock which was described in ENGELS and PRESTON (1979), and was obtained from J. LUCCHESI (University of North Carolina).

Genetic crosses: The selection for new mutations at *Tpl* was carried out as described in Figure 1 and below. The dysgenic hybrids were raised at 23°, while the subsequent crosses were at 20°. For the selection cross, vials were set with 20 pairs of flies which were turned into fresh vials every 2 days. The number of zygotes produced in cross 1 was estimated by counting the total progeny from 15 *C(1)M3,y² bb/Y;;Dp(Tpl) p^p/Df(Tpl) p^p* females and mated to *Y^S X.Y^L, In(1)EN y/Y;;Dp(Tpl) p^p/Df(Tpl) p^p* males and multiplying by two to account for the inviable *Dp(Tpl)/Dp(Tpl)* and *Df(Tpl)/Df(Tpl)* zygotes. For estimating the zygotes produced in cross 2, the progeny of 20 dysgenic F_1 females mated to their male siblings were counted.

Hybridization *in situ* to polytene chromosomes: Salivary gland squashes for *in situ* hybridization were prepared essentially as described in ENGELS *et al.* (1986), except that slides were not pretreated with Denhardt's solution. Plasmid pπ25.7wc DNA (KARESS and RUBIN 1984), a gift from J. LUCCHESI (University of North Carolina), was biotinylated by nick translation with bio-16-dUTP (Bethesda Research Labs) substituted for dTTP. Hybridizations were carried out at 37° in a buffer containing 4 × SSPE, 40% formamide and 1 μg/ml salmon sperm DNA (Sigma). The Enzo Biochemicals Detek-1-HRP kit was used for peroxidase staining as described in ASHBURNER (1989).

RESULTS

Recovery of mutations: F_1 flies from a dysgenic cross between π_2 males and Oregon-R females were collected. In cross 1, dysgenic males were mated to *C(1)M3 y² bb/Y;;Dp(Tpl) p^p/Df(Tpl) p^p* females, while in cross 2, dysgenic females were mated to *Y^S X.Y^L, In(1)EN y/Y;;Dp(Tpl) p^p/Df(Tpl) p^p* males as outlined in Figure 1. All F_2 progeny from these crosses were expected to die unless a new mutation or rearrangement involving *Tpl* had occurred. The p^+ allele on the mutagenized chromosome allowed the new *Tpl* alleles to be followed during test crosses and during backcrossing to *Dp(Tpl) p^p/Df(Tpl) p^p* flies. The different types of F_2 survivors arising from these two crosses are listed in Table 1. The genotypes of some of the survivors could not be tested due to infertility or a short adult life span. Four of the progeny of cross 1 were triploids. An additional 19 survivors each had a *Tpl⁺ p^p* chromosome, presumably resulting from recombination between the *Dp(Tpl) p^p* and *Df(Tpl) p^p* chromosomes (DENELL 1976). Neither of these kinds of progeny resulted from cross 2.

As can be seen in Table 1, we recovered a large number of new duplications of *Tpl* from these crosses. The 19 duplications listed were recovered from 12 different mating vials, and thus represent at least 12 independent mutational events. Some of these had very poor viability and have been lost. The breakpoints of the rearrangements in those duplications we were able to analyze are illustrated in Figure 2. Of the duplications that we were able to characterize, all but one appear to be direct tandem repeats with one or both breakpoints often corresponding to the location of *P* elements in the parental π_2 stock (Figure 3, A, B, C and D, and other data not shown). The one exception, *Dp(3;3)Tpl^J25*, appears to be a reverse tandem duplication. As would be expected, tandem direct duplications are not completely stable and many of these were also lost subsequent to the cytological analysis.

Fifteen of the new *Tpl* mutations were found to complement duplications of the locus. These 15 mutations represent at least 10 independent events. All of these alleles appear to have normal cytology, and are viable when heterozygous with wild-type third chromosomes. They are thus genetically similar to the previously described class of hypomorphic mutations which includes the alleles *Tpl^10*, *Tpl^17* and *Tpl^38* (ROEHRDANZ and LUCCHESI 1980). None of the 15 new hypomorphic mutations complement the *Tpl^10* allele for the recessive lethal phenotype. Of the 6.3×10^4 and 8.1×10^4 zygotes estimated for crosses 1 and 2, respectively, only half (those carrying *Dp(3;3)21l73*) would allow the recovery of new hypomorphic mutations. The recovery of 15 hypomorphic alleles gives an estimated mutation rate of 2

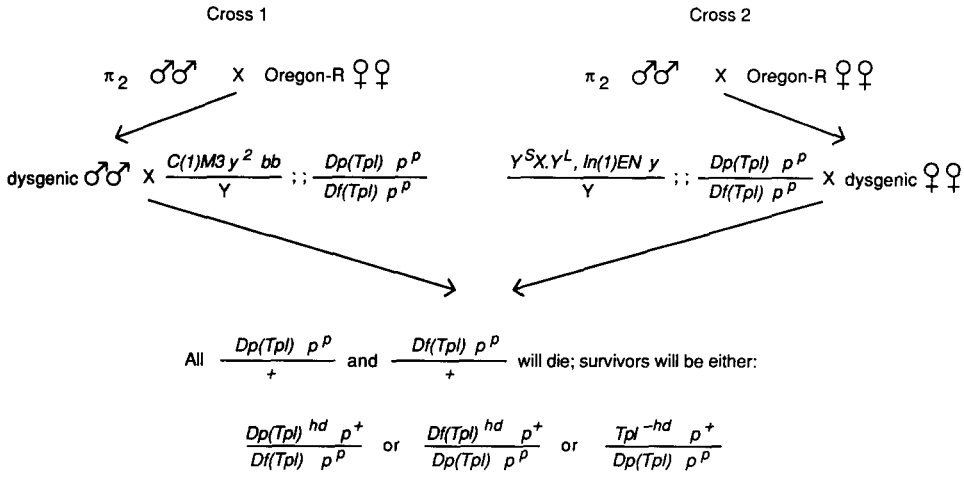

FIGURE 1.—Genetic selection for mutations of *Tpl*. Males from the π_2 stock were mated to Oregon-R virgin females. Both male and female dysgenic F_1 progeny were used in reciprocal crosses to *Dp(Tpl) p^p/Df(Tpl) p^p* flies as shown. The occasional p^+ F_2 survivors were backcrossed to *Dp(Tpl) p^p/Df(Tpl) p^p*. The p^+ F_3 progeny were tested by crossing to *ri Tpl^10 Ki p^p/TM3, Ser* flies. When the mutagenized chromosome carried a new duplication, then only *Dp(Tpl)^hd p+/ri Tpl^10 Ki p^p* progeny survived in the test cross, and a stock was established from the F_3 progeny. If the mutagenzied chromosome had carried a new deficiency or null mutation, then only *Df(Tpl)^hd p+/Dp(Tpl) p^p* would have survived and been used to establish a stock (we did not recover this class). If a new hypomorphic allele resulted, the test cross resulted in both *Dp(Tpl) p^p/ri Tpl^10 Ki p^p* progeny which were discarded, and *Tpl^-hd/TM3, Ser* progeny which were used to establish a balanced stock. The second and fourth chromosomes are all wild type and are not shown.

TABLE 1

Exceptional progeny recovered

Genotypes of surviving progeny	Number of survivors	
	Cross 1	Cross 2
$\frac{Dp(Tpl)^{hd}\ p^+}{Df(Tpl)\ p^p}$	7	12
$\frac{Df(Tpl)^{hd}\ p^+}{Dp(Tpl)\ p^p}$	0	0
$\frac{Tpl^{-hd}\ p^+}{Dp(Tpl)\ p^p}$	2	13
$\frac{Tpl^+\ p^p}{Tpl^+\ p^+}$ (recombinants)	19	0
Triploids (females or intersexes)	4	0
Unknown (infertile or lost)	5	21
Total	37	46

Crosses were carried out as described in the text and Figure 1. The first three rows indicate new mutational events involving *Tpl*, the next three rows are other genetic events that led to exceptional progeny.

$\times 10^{-4}$, significantly higher than the rate of recovery of hypomorphic mutations following chemical mutagenesis (KEPPY and DENELL 1979; ROEHRDANZ and LUCCHESI 1980). We have previously shown that *Tpl^10*, *Tpl^17* and *Tpl^38* are all associated with a site-specific increase in recombination frequency and with premeiotic recombination in males (DORER and CHRISTENSEN 1989). These observations led us to propose that insertions of transposable elements could be responsible for the phenotypes of these mutations. That we have now recovered new hypomorphic alleles at a high rate following hybrid dysgenesis supports this hypothesis.

Analysis of hypomorphic alleles: For 14 of the 15 new hypomorphic alleles, *in situ* hybridization with a *P* element probe showed no hybridization signal at 83DE, although numerous other sites of hybridization were clearly visible (Figure 3, E and F; other data not shown). One mutant, *Tpl^J34*, shows a positive hybridization signal at 83E (Figure 4A). In an attempt to determine whether the *P* element at 83E segregated with the hypomorphic phenotype of *Tpl^J34*, a recombinant chromosome of the genotype *ri+ Tpl^J34 Ki p^p* was recovered from a female of the genotype *ri+ Tpl^J34 Ki+ p+/ri Tpl+ Ki p^p*. This chromosome still carries the *Tpl* hypomorphic phenotype, but it no longer has a *P* element hybridization signal at 83E (Figure 4B). Since we have recovered only one such recombinant, it is difficult to rule out alternative possibilities, but this result suggests that the *P* element inserted at 83E is probably not responsible for the mutant phenotype.

Reversion of hypomorphic alleles: In the course of maintaining the hypomorphic mutations as stocks balanced with *TM3, Ser* for several generations we occasionally observe flies who do not carry the *TM3* chromosome. Further crossing and testing of these exceptional flies reveals that they are heterozygotes carrying one wild-type chromosome and one mutant chromosome. We also made lines from single mutant

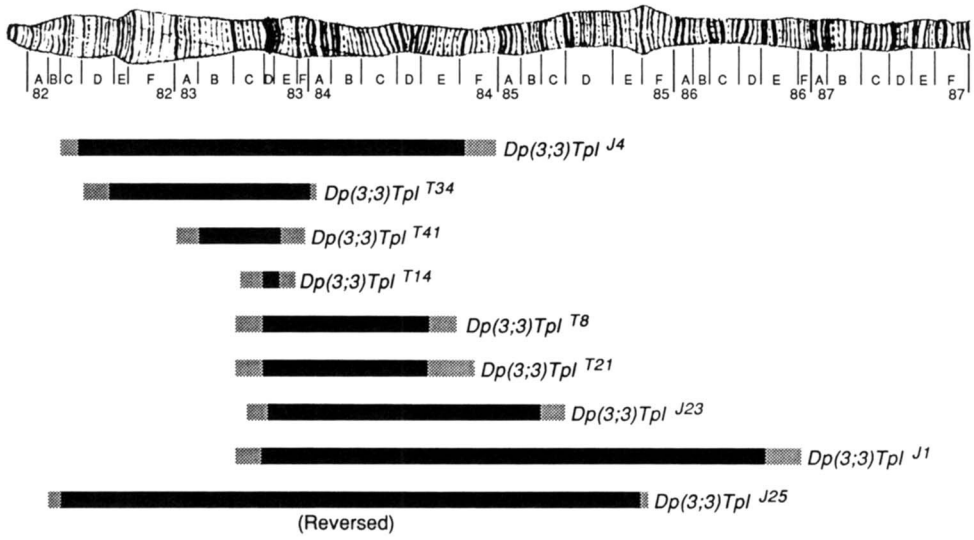

FIGURE 2.—Cytological extent of duplications recovered. The extent of duplications recovered is shown aligned with BRIDGES' (1935) map. All are tandem direct duplications except *Dp(3;3)Tpl*^{J25} which is a tandem reverse duplication. The gray areas of the bars represent uncertainties in the determination of the breakpoints.

flies, which bred true for several generations, and then were able to recover revertants in the stock. The revertant lines are always stable. These revertants therefore must actually arise as spontaneous events rather than being due to a second-site suppressor that segregates. We have also systematically looked for revertants of the hypomorphic alleles by crossing large numbers of *Tpl*^{hd}*/TM3,Ser* flies to *ri Tpl*^{10} *Ki p*^{p}*/TM3, Ser*. Any Ki Ser^{+} progeny that result are tested and generally found to be revertants of the dysgenic hypomorphic allele. Some of our new hypomorphic alleles are quite stable, while others are occasionally seen to revert. We have established lines from several of these revertants, but since we do not know the basis of the mutations, we are as yet unable to determine the basis for reversion. We have never observed revertants among the original three hypomorphs from the ROEHRDANZ and LUCCHESI (1980) study, *Tpl*^{10}, *Tpl*^{17} and *Tpl*^{38}.

DISCUSSION

In general one expects mutations induced by hybrid dysgenesis to be due to insertions of the *P* element into a gene. Although there have been occasional reports of P-M hybrid dysgenesis resulting in transposition of other elements (RUBIN, KIDWELL and BINGHAM 1982; LEWIS and BROOKFIELD 1987), ENGELS has presented data suggesting that P-M hybrid dysgenesis does not mobilize other elements (EGGLESTON, JOHNSON-SCHLITZ and ENGELS 1988; ENGELS 1989). While the *P* transposase is probably very specific for *P* element sequences, very little is known about the control of transposition of other elements. Thus it is certainly possible that when π_2 males are crossed to Oregon-R females, other transposable element systems may also be activated. In this study we used this dysgenic cross to generate mutations and rearrangements involving the *Triplo-lethal* locus. Interestingly, although we obtained evidence that *P* elements were mobilized, at least one other element was likely to have been mobilized to a high degree in the cross.

The duplications we recovered are consistent with the findings of ENGELS and PRESTON (1984). In that study they showed that most of the rearrangements recovered following hybrid dysgenesis had breakpoints that coincided with the locations of *P* elements in the parental strain, and that *P* element sequences often remained at the new junctions created by the rearrangement. Many of the duplications we recovered have breakpoints in common with the locations of *P* elements in our π_2 stock (data not shown). Many of the duplications also had *P* element sequences remaining at a breakpoint (Figure 3, A, B, C and D, and other data not shown). The simplest way to explain the genesis of our duplications is by a two-break interchromosomal event, with at least one of the breaks usually occurring at the site of a *P* element. The reverse tandem duplication, *Dp(3;3)Tpl*^{J25}, is most likely the result of a three-break event. A transposition would also have required a three-break event and would have been expected to occur at lower frequencies, so the absence of this class is not surprising. Deficiencies would most likely have been produced by a similar two-break process. However, this would often have produced large deficiencies which would either have uncovered a haplo-inviable region nearby [LINDSLEY *et al.* (1972) report haplo-inviability for 82C to 83E and 84D through 85D] or uncovered a recessive lethal at one of the breakpoints of *Dp(3;3)21l73* (KEPPY and DENELL 1979).

In view of this evidence that the π_2 × Oregon-R cross mobilized *P* elements, the data on the hypomorphic alleles of *Tpl* are surprising. Previous workers have found that the rate of mutation at *Tpl* is very low, even in the presence of chemical mutagens such as EMS (KEPPY and DENELL 1979; ROEHRDANZ and LUCCHESI 1980). Given that we have recovered hy-

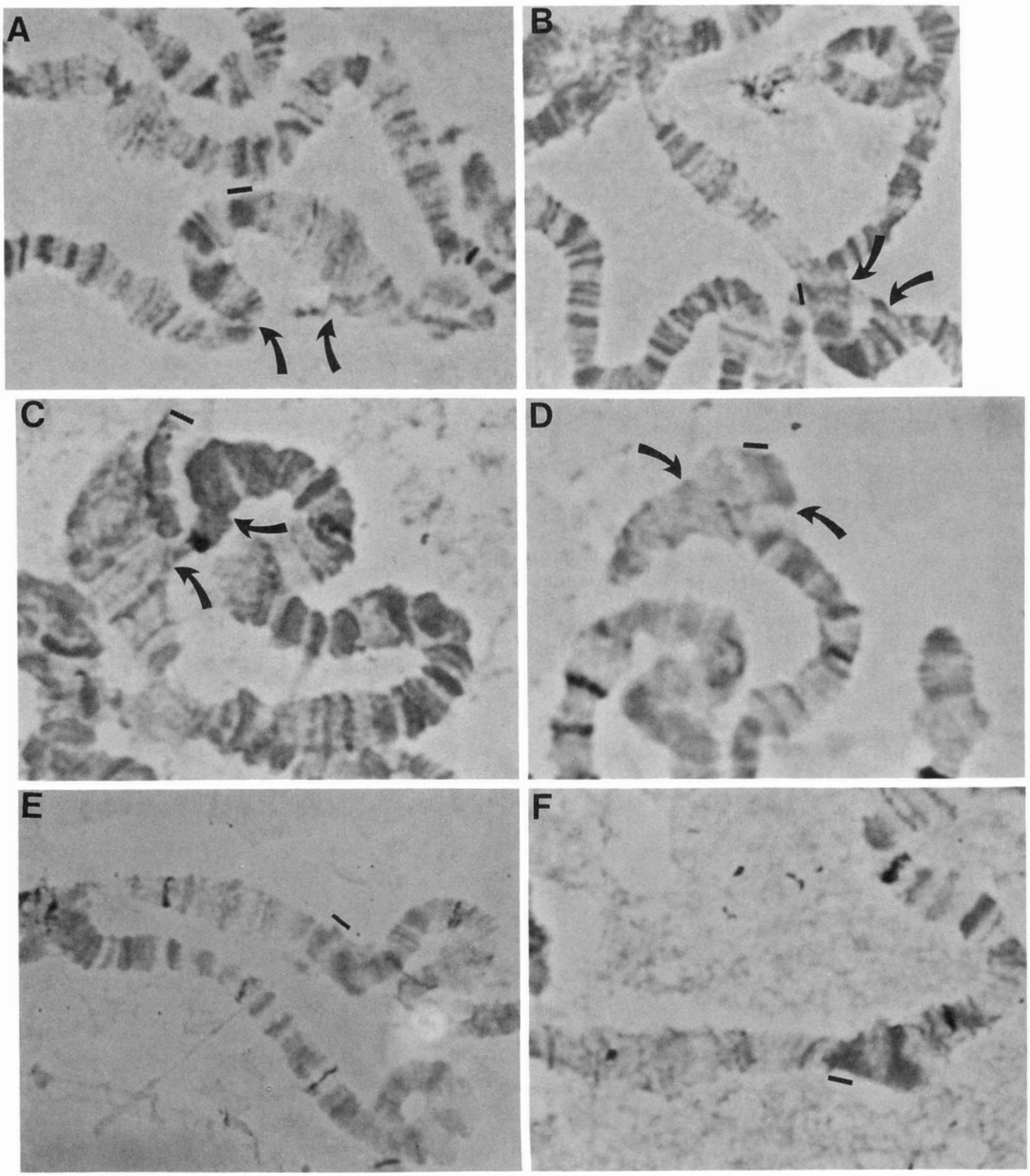

FIGURE 3.—*In situ* hybridizations of selected duplications and hypomorphic mutations. Polytene chromosomes were squashed, hybridized *in situ* with a biotinylated *P* element probe and stained with Giemsa as described in MATERIALS AND METHODS. The 83DE bands are indicated by a bar. Panels A, B, C and D are new duplications of *Tpl*; as follows: (A) $Dp(3;3)Tpl^{J4}/Df(3R)18i77$, (B) $Dp(3;3)Tpl^{T8}/Df(3R)18i77$, (C) $Dp(3;3)Tpl^{T34}/Df(3R)18i77$, and (D) $Dp(3;3)Tpl^{T41}/Df(3R)18i77$. Arrows indicate the breakpoints of the duplications. Panels E and F are hypomorphic alleles, as follows: (E) $Tpl^{J36}/Dp(3;3)21l73$ and (F) $Tpl^{T37}/Dp(3;3)21l73$.

pomorphic mutations in *Tpl* at a rate significantly higher than that seen in any other study, it seems likely that these mutations were the result of the dysgenesis we induced. Furthermore, the instability of some of these mutations suggests that they are due to transposon insertions. However, none are due to *P* element insertions that are detectable by *in situ* hybridization. Other dysgenic systems are known in

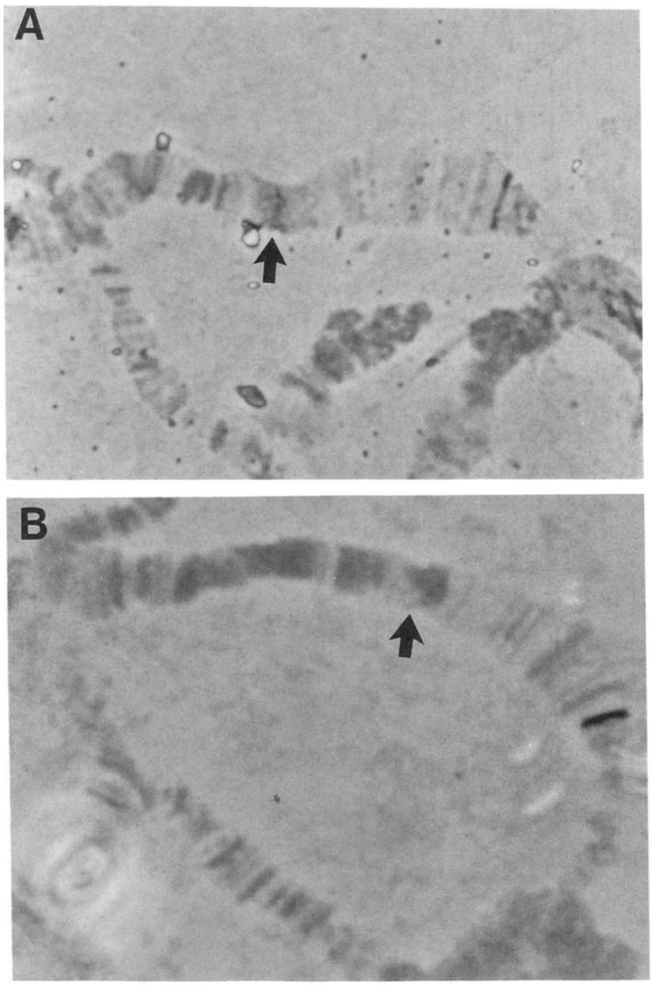

FIGURE 4.—*In situ* hybridization of the hypomorphic allele Tpl^{J34}, and a Tpl^{J34} *Ki* p^{p} recombinant chromosome. Polytene chromosomes were squashed, hybridized *in situ* with a biotinylated *P* element probe and stained with Giemsa as described in MATERIALS AND METHODS. The probe was pπ25.7wc. Panel A is $Tpl^{J34}/TM3$, *Ser*, and panel B is the recombinant described in the text, Tpl^{J34} *Ki* $p^{p}/TM3$, *Ser*. The 83E region is indicated by an arrow.

Drosophila melanogaster, including the I-R system (FINNEGAN 1989) and *hobo* (BLACKMAN and GELBART 1989). The π_2 × Oregon-R cross has apparently not been widely used for *P* element hybrid dysgenesis so it is difficult to know whether either of these other dysgenic systems or an as yet undescribed one was activated in the cross.

Finally, our data have implications for the structure of the *Triplo-lethal* locus. Eighteen years have passed since the locus was first described (LINDSLEY *et al.* 1972) and the genetics has become only more enigmatic. It seems to be nearly impossible to recover complete inactivations of the locus that are not deletions. Two alleles did result from the mutagenesis carried out by KEPPY and DENELL (1979) which behave genetically as deficiencies but are cytologically normal. It is possible that these are small deficiencies that cannot be seen in polytene preparations, or they may in fact represent the extremely rare occurrence of true point mutations. KEPPY and DENELL (1979) suggested three possible genetic explanations to account for the paucity of null mutations: 1) the locus, and hence the target size for single base-pair changes is very small; 2) the locus may not encode a protein and therefore is not readily disrupted by single base-pair changes; or 3) the locus is redundant in some way, possibly encoding two or more proteins with overlapping or identical functions.

It is paradoxical that deletions are readily selected (KEPPY and DENELL 1979; ROEHRDANZ and LUCCHESI 1980), and hypomorphs are readily recovered following hybrid dysgenesis, but point mutations are seemingly never recovered. If the locus is redundant, and inactivating one of two or more equivalent genes gives a hypomorphic phenotype, then why should single base-pair changes fail to inactivate one gene and also give a hypomorphic phenotype? If hypomorphs are due to partial inactivations, then why do we fail to recover complete inactivations that are due to transposon insertions? There must be some fundamental difference in the molecular effects of single base-pair changes *vs.* transposon insertions to which *Tpl* is peculiarly sensitive. Point mutations would most likely result in a missense protein while transposon insertions might be more likely to affect the rate of accumulation of a correctly processed and translatable mRNA. This difference might account for the unusual genetics of *Tpl*. It is our hope that the forthcoming molecular analysis (D. R. DORER, A. C. CHRISTENSEN and D. H. JOHNSON, in preparation) will shed some light on this strange and unique locus.

This work was inspired by the late LARRY SANDLER who, in June 1983, suggested to A.C.C. that it would be "easy" to get *P* element-induced mutations of *Tpl* by hybrid dysgenesis. We are grateful to the inscrutable MARILYN CADDEN for technical assistance, and to JOHN C. LUCCHESI for interesting discussions and for stocks. We thank BRIAN MARIANI for helpful comments on the manuscript, and DANIEL JOHNSON for being enthusiastic about our data. This work was supported in part by National Institutes of Health grant R29-GM38483. D.R.D. was supported in part by a predoctoral fellowship from the Percival E. and Ethel Brown Foerderer Foundation.

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