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Genetics

mu-2: Mutator gene in *Drosophila* that potentiates the induction of terminal deficiencies

(x-ray-induced deletions/telomere/DNA repair)

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ABSTRACT **An x-ray-dependent mutator on chromosome 3 of *Drosophila melanogaster* is described that specifically increases the recovery of deletions for chromosomal tip regions. Such deficiencies can be induced on any chromosome. More centromere proximal mutations, as assayed by the sex-linked recessive lethal test, are not increased over the wild-type control. As far as can be determined by genetic, cytological, and molecular assays, the deletions extend to the very end of the chromosome involved. In addition, the frequency of these deletions is directly proportional to x-ray dose, suggesting that they are one-break rearrangements. It is proposed that the mutator is blocked in a major pathway for the repair of DNA double-strand breaks, and that a minor repair pathway is responsible for the addition of new telomeres under these conditions.**

Early observations by Muller and co-workers (1–3) indicate that broken chromosome ends tend to fuse with one another. Fusion of ends produced by two or more different breaks would lead to chromosome aberrations—e.g., translocations or deletions. Observations on the recovery of broken chromosomes indicate that a broken end may fuse with another broken end but not with an unbroken end. Thus, terminal inversions are never recovered. In addition, terminal deficiencies are not recovered; presumptive terminal deletions are capped with a normal chromosome end—its own or that from another chromosome. These findings led Muller (2, 3) to propose that a special structure, the telomere, exists at the tips of all chromosomes to prevent the termini from fusing with each other and, thus, to maintain chromosome stability. There is now ample molecular evidence indicating that telomere regions represent distinct structural domains, portions of which are evolutionarily conserved (4–11), and that these play an essential role in preserving the stability and structural integrity of linear chromosomes (6, 11, 12).

There have been a number of reports over the years of the recovery of terminal deficiencies (13–15) and translocations (14, 16, 17). The most extensive observations were made by McClintock (18–20), who showed that in maize endosperm a single broken chromatid end may replicate and fuse with its sister chromatid broken end to produce a dicentric chromosome, which will form a bridge at the next anaphase. Subsequently, the bridges break to repeat this bridge–breakage–fusion cycle. The same broken chromosome will "heal" at about the time of the first mitotic cycle in the embryo and can be recovered as a stable terminal deficiency. These observations raise the possibility that, under some conditions, broken uncapped chromosome ends can be recovered. In *Drosophila*, however, this occurrence is extremely rare (21).

There are as yet no clear, unambiguous criteria to prove that a putative terminal deletion is not capped by a previously existing telomere. Three tests have been suggested, but none tests directly for the presence of an added telomere. The polytene chromosomes of *Drosophila* can be examined in detail for the presence of material distal to a break. Genetic markers also have been used to identify the presence of specific chromosome ends (1). Recently, a cloned *Drosophila melanogaster* DNA sequence has been identified that *in situ* hybridizes specifically to the tips of *D. melanogaster* polytene chromosomes and can be used to mark tip regions (4).

We describe here a mutator gene (symbol: *mu-2*) that maps to the third chromosome of *D. melanogaster* and that potentiates the production of presumptive terminal deletions among the gametes of homozygous females exposed to a relatively low dose of x-rays. The frequency of these deletions, most readily assayed on the X chromosome, increases in direct proportion to the dose of x-rays applied, suggesting that they are simple one-break chromosome aberrations. As judged by cytological, genetic, and molecular criteria, the deletions are terminal. However, the overall frequency of x-ray-induced lethal mutations is not increased over that of wild-type controls. We interpret these results to mean that *mu-2* is defective in the repair of x-ray-induced chromosome breaks, thus allowing the healing of broken chromosome ends.

MATERIALS AND METHODS

Table 1 includes a short description of several mutants and specific chromosomes which were used. A more complete description is given by Lindsley and Grell (22) and Lefevre (23).

In most of the experiments described, wild-type and homozygous *mu-2* females were collected, held 1–4 days, and exposed to 500 roentgen (R) of 150 kV x-rays. They then were crossed individually to one of two tester males (either *y w*/*y*^{2} *sc·Y* or *y*/*y*^{2} *sc·Y*; *net*; *ru*; *spa*^{pol}) for 3 days, after which all parents were discarded. The progeny were scored for the expression of the recessive paternal markers, and stocks were made of the mutants. Newly arisen *y* mutations were kept in males over the *y*^{2} *sc·Y* duplication balanced with *C(1)DX*, *y f* females.

In the dose–response experiment, females age 3–5 days were treated with 150 kV x-rays with 1 mm of Al filter, mated *en masse* to *y w*/*y*^{2} *sc·Y* males for 22–24 hr, and then discarded. These changes allow a more uniform population of stage 14 oocytes to be tested.

Salivary gland polytene chromosomes were prepared from Canton S, Oregon R, and *mu-2* stocks of *D. melanogaster*. The *y*$^-$ mutant X chromosomes derived from irradiated *mu-2* or Oregon R females were examined cytogenetically in either *y*/*y*^{2} *sc·*Y males or the female progeny resulting from mating *y*/*y*^{2} *sc·*Y males with Canton S females. *In situ* hybridization

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Abbreviations: kb, kilobase(s); 2L and 2R, 3L and 3R, etc., left and right arms of the second and third chromosomes, respectively.

Table 1. Synopsis of mutants used in text

Mutant symbol	Phenotype	Linkage
l(1)EC1	Lethal	X–0.0
y	Yellow body color	X–0.0
y^2	Yellow-2 body color	
ac	Achaete bristles	X–0.0
sc	Scute bristles	X–0.0
M(1)Bld	Minute bristles	X–0.1
pn	Prune eye color	X–0.8
w	White eye color	X–1.5
f	Forked bristles	X–56.7
C(1)DX	Attached X chromosome	
net	Net wing veins	II–0.0
b	Black body color	II–48.5
sp	Speck wing	II–107.0
ru	Roughoid eyes	III–0.0
ve	Veinlet wings	III–0.2
st	Scarlet eye color	III–44.0
ca	Claret eye color	III–100.7
TM3	Third chromosome balancer	Multiple inversions
spa^{pol}	Sparkling-poliert eyes	IV–

experiments also were carried out on the above stocks.

Chromosomes were prepared by the method of Lim and Snyder (24), except that the larvae were dissected in 45% acetic acid. Permanent slides were made by using Zeiss L15 mounting medium. The *in situ* hybridization procedure, including preparation of salivary gland chromosomes, has been described (25). Telomere-region probe DNA was prepared by nick-translation of plasmid pDm3.0, which contains a single 3.0-kilobase (kb) repeat of cDm356 (4) inserted into the *Bam*HI site of pBR322.

RESULTS

When females carrying two genetically wild-type X chromosomes are crossed to $y\ w/y^2\ sc{\cdot}Y$ males, the regular male and female progeny are phenotypically wild type. However, mutations or deletions occurring at either the *y* or *w* locus of the maternal X chromosome will give rise to progeny expressing the appropriate phenotype. Table 2 gives the results from such crosses. In the absence of radiation, both *mu-2* and Oregon R produced *y* and *w* mutations at a low frequency. In both strains the frequency of *w* mutations was similar to the frequency observed by Schalet (as cited in ref. 26) in a large-scale study of spontaneous mutation. Similarly, the *y* mutation frequency in Oregon R was the same as that observed by Schalet. However, the spontaneous mutation frequency at the *y* locus in *mu-2* females was increased significantly over the wild-type control. X-irradiation greatly enhanced the efficiency of the recovery of *y*, but not *w*, mutations from *mu-2* females. After irradiation with an x-ray dose of 500 R, the frequency of *y* mutants recovered among the progeny of irradiated *mu-2* females was an order of magnitude greater than the frequency of *y* mutants recovered from untreated wild-type females. However, there was no difference in the frequency of recovered *w* mutants. This finding suggests that *mu-2* promotes terminal deletions (scored as *y* mutants) and not interstitial deletions (scored as *w* mutants).

Table 2. Mutations recovered among the progeny of females with wild-type X chromosomes crossed to $y\ w/y^2\ sc{\cdot}Y$ tester males

Maternal genotype	X-ray dose, R	Progeny +	y*	% y	w	% w
mu-2/mu-2	500	39788	43	0.11	3	0.01
mu-2/mu-2	0	35020	4	0.01	0	—
+/+	500	77528	6	0.01	4	0.01
+/+	0	54394	2	0.00	1	0.00

*"y" is the sum of y females, y^2 males, and y^2 *sc* males.

Site Specificity. The specificity of *mu-2* was examined further in two experiments. In the first, the production of x-ray-induced sex-linked recessive lethals in *mu-2* and wild-type females was compared. There was no difference between the mutant and the control (Table 3); in both cases the recessive lethal frequency approximated 1.3%. Although no effort was made to eliminate previously existing lethals in these stocks, the progeny from individual females were kept together so that large clusters would be evident; no clusters were found.

The genetic analysis of the *y* mutants derived from irradiated *mu-2* females confirmed the inference that they are deletions. Of 41 *y* mutations tested, 39 were also mutant for the neighboring locus *sc* (Table 2). Of the remaining two, one was hemizygous viable, and thus was probably a mutation limited to the *y* locus. The other was a deficiency with a breakpoint between *y* and *sc* (see below). Six *y* mutations were recovered from the wild-type control. One of these was hemizygous viable, suggesting it is a point mutation. Two others carried the wild-type allele for *sc*, although they were deficient for another nearby locus (*l(1)EC1*; see below). The remainder were deficient for *sc*. Thus, five of the six *y* mutations from the control appear to be small deficiencies.

As a genetic test for the presence of the *X* chromosome tip, the lethal-bearing *y* mutants were tested for complementation with a lethal mutation of the left-most known locus, *l(1)EC1*. Males carrying a *y* deletion (y^-) and the $y^2\ sc{\cdot}Y$ chromosome were crossed to *l(1)EC1/y pn* females, and the progeny were scored for the recovery of y^+ females ($y^-/l(1)EC1$) relative to their *y* ($y^-/y\ pn$) siblings. Surviving y^+ females were progeny-tested for the presence of a $y^2\ sc{\cdot}Y$ chromosome. In no case did euploid y^+ females survive. Thus, all of the lethal-bearing *y* mutations recovered are functionally deficient for *l(1)EC1*, the locus closest to the tip of the *X* chromosome.

In a similar series of tests, the ability of the y^- chromosomes to "cover" autosomal tip markers was determined. Males of the genotype $y^-/y^2\ sc{\cdot}Y$ were crossed with *C(1)DX*, $y\ f/y^2\ sc{\cdot}Y$ females homozygous for the appropriate tip markers on one autosome (*net b sp* or *ve st ca* or spa^{pol}), F_1 males were backcrossed, and F_2 males were examined for the expression of tip markers. [Note: *net* marks the tip of the left (L) arm of the second chromosome, 2L; *sp*, the tip of the right (R) arm of the second chromosome, 2R; *ve*, the tip of 3L; *ca*, the tip of 3R; and spa^{pol}, the tip of 4R.] If males expressed the mutant phenotype for a tip marker, it was determined that the y^- chromosome did not carry the wild-type allele of that locus. None of the y^- chromosomes carried the wild-type allele of any of these tip markers.

Thirty of the induced lethal *y* mutations derived from irradiated *mu-2* females were examined cytologically. There was no cytological evidence that these deficiencies are capped by autosomal material (Fig. 1). In preliminary experiments, G. Lefevre (personal communication) also could not see the chromosome tip in *y* deletions arising spontaneously in *mu-2*. On this point the cytological evidence confirms and extends the genetic data because the genetic markers that were used are not located at the extreme ends of their respective arms. A translocation involving only a few bands would be

Table 3. Sex-linked recessive lethals produced by irradiation of females with 500 R of x-rays

Genotype of treated female	Total X chromosomes	Lethal-bearing chromosomes	% lethal
mu-2/mu-2	1186	16	1.37
+/+	479	6	1.27

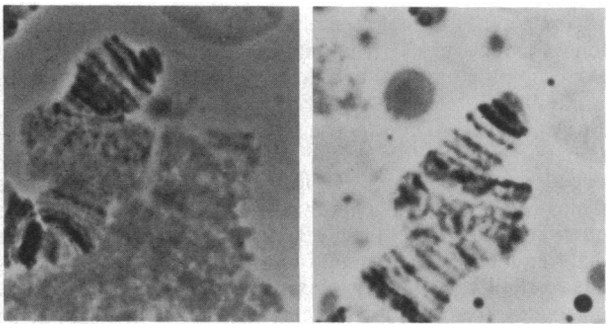

FIG. 1. X chromosome tips of two females heterozygous for terminal deficiencies and a wild-type X chromosome from Canton S. (*Left*) RT96/+. (*Right*) RT13/+.

evident cytologically. Such translocations were not found. Rather, each deletion extended from the tip to a breakpoint in the 1B–1E region. Deficiencies extending further were not expected to be recovered in F_1 females because large heterozygous deficiencies have low viabilities (27) and because such deficiencies would uncover *M(1)Bld*, a haplosensitive locus (see ref. 22). In the limited number of deficiencies examined here, the breakpoints were distributed throughout the 1B–1E region; there was no evidence for a hot spot.

Twelve of the y^- deficiencies recovered from *mu-2* females were examined for their ability to hybridize to the 3-kb cDm356 sequence, which hybridizes specifically to tip regions (4). None of the deficiencies showed any hybridization with this probe (Fig. 2). In contrast, the X chromosome of the parental *mu-2* and Oregon R stocks exhibited significant labeling at their tip regions when probed with the 3-kb telomere sequence.

The occurrence of deletions was not restricted to the tip of the X chromosome. Deletions of autosomal tips also were recovered when females were treated with x-rays and crossed to homozygous y/y^2 *sc·Y*; *net*; *ru*; spa^{pol} males. The latter three markers map near the tips of 2L, 3L, and 4R. Mutations at the four tips were recovered with comparable frequencies from *mu-2* females (Table 4). Their frequency was ≈10 times higher in *mu-2* females than in the Oregon R control. Several of the presumptive autosomal deletions arising from *mu-2* females were tested for viability; all proved to be lethal when homozygous, confirming that they are deletions. These observations show that, in the presence of *mu-2*, the x-ray-induced frequency of deletions at all chromosome tips is increased dramatically.

Dose–Response. If the *y* mutations derived from *mu-2* females were terminal deletions, the frequency of these mutations would increase linearly with the dose of x-rays. However, if such mutations were interstitial deficiencies or reciprocal translocations, they would require two chromosome breaks. Thus, their frequency would increase exponentially

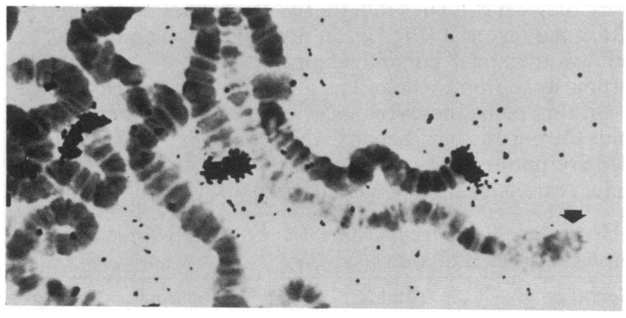

FIG. 2. Chromosomes of a male bearing the terminal deficiency RT26 hybridized *in situ* with 3-kb cDm 356 sequences. The deficient X chromosome tip is indicated by an arrow.

Table 4. Mutations induced at the tips of the four chromosomes of females irradiated with 500 R of x-rays*

Maternal genotype	Progeny				
	+	*y*	*net*	*ru*	spa^{pol}
mu-2/mu-2	15,179	9	2	8	11
+/+	13,762	1	1	1	0

*The markers used are near the tips of the X, 2L, 3L, and 4R chromosomes, respectively.

with the dose. Empirically, two-break rearrangements have been observed to increase with a power of 3/2. To distinguish between these two possibilities, *mu-2* females were treated with various doses of x-rays, up to 1000 R, and the mutation frequency was determined at each dose. As can be seen in Table 5 and Fig. 3*A*, the frequency of *y* deletions increased linearly with the dose, consistent with the idea that they are terminal deletions resulting from a one-break event.

The *w* males recovered in these experiments (Table 5; Fig. 3*B*), however, probably derive from two-break rearrangements. The evidence for this was 3-fold. First, no complementary *w* females were recovered, suggesting that these *w* males do not result from point mutations or small interstitial deficiencies. Second, all of these males were sterile as would be expected if they were X/0 males carrying a small duplication of the X chromosome tip, including the y^+ locus. Third, the frequency of these males increases with the 1.5 power of the dose of x-rays (Fig. 3*B*), suggesting that more than one chromosome break is required for their production.

Although the *y w* males were the result of chromosome loss, the cause of this loss is uncertain. It may be due, to some extent, to chromosome breakage because the frequency increased with a power of 1.6 (Fig. 3*B*) as the x-ray dose was increased. In any case, at least some chromosomal rearrangements (e.g., those producing *w* males) increased with two-hit kinetics. This supports the argument that the linear response of *y* deletions to x-ray dose is due to one-hit events.

Linkage. Linkage studies were undertaken to determine the genetic nature of the factors controlling the frequency of these deficiencies. Each of the three major chromosomes was extracted from the original strain and put into a new balanced stock in which the two other chromosomes were derived from a mu^+ strain. Then each of the major chromosomes could be tested independently of the others for mutator activity. The X and second chromosomes from the original *mu-2* stock did not carry mutator activity. Females homozygous for the third chromosome, however, produced a high frequency of *y* mutations. Heterozygous *mu-2/TM3* females produced control levels of mutator activity, indicating that *mu-2* is recessive. In addition, *mu-2* mapped as a single locus very close to *ru* near the tip of 3L (unpublished data).

Even though *mu-2* was responsible for the recovery of stable terminal deficiencies, it was not required in order to

Table 5. Mutational response of irradiated females to x-ray dosage

Maternal genotype	X-ray dose, R	Progeny			
		+	*y w*♂	*y*	*w*
mu-2	0	54348	272	7	0
	100	24844	233	21	0
	200	19612	426	25	6
	400	11044	550	34	4
	500	10565	731	35	11
	800	6130	909	29	14
	1000	83	26	0	0
Ore-R	0	54394	40	2	1
	500	23793	163	4	13

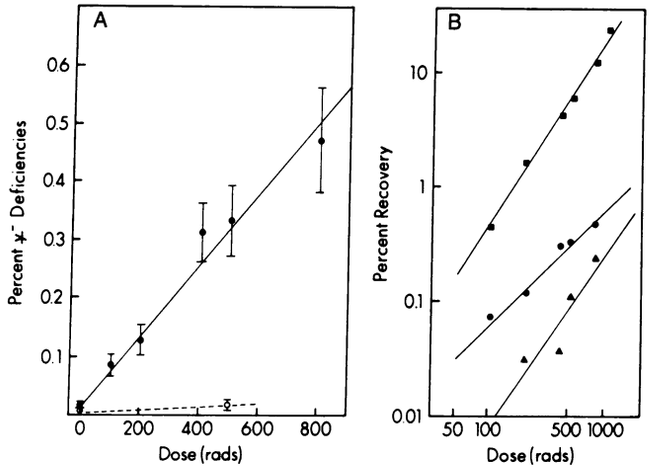

FIG. 3. The response of the frequency of *y* deficiencies to the x-ray dose. Females with wild-type X chromosomes were irradiated and mated to *y w*/y^2 *sc·Y* tester males. The progeny were scored phenotypically to characterize the genotypes of the irradiated maternal X chromosomes, and the data are presented in Table 6. (*A*) The frequency of *y* deficiencies from *mu-2* (●) and Oregon R (○) females. Bars represent standard errors. (*B*) A log-log plot of the frequencies of recovered exceptional progeny: ■, X/0 (*y w*) males; ●, yellow (*y*) mutations; ▲, white (*w*) mutations. The slopes are 1.6, 1.0, and 1.5, respectively.

maintain the stability of these deficiencies. The progeny in which the *y* mutations were recovered resulted from a cross of *mu-2* females by *mu-2*$^+$ males and, thus, were heterozygous for the locus. In order to establish a stock, the F_1 y^-/y^2 *sc·Y* males are outcrossed once and y^-/y *w* females are outcrossed twice, effectively diluting out the *mu-2* present in P_1 females. Since *mu-2* is recessive, the terminal deficiencies could not be recovered if *mu-2* were responsible for maintaining their stability. Some of these deficiencies have been maintained in stock for 4 years.

DISCUSSION

In the presence of *mu-2*, a high frequency of mutations in the tip regions of all four chromosomes is induced by a relatively low dose of x-rays. Virtually all of the X chromosome mutations are deletions that uncover *l(1)EC1*, the most terminal known locus. None of the deficiencies show either genetic or cytological evidence of being capped by an autosomal telomere. In addition, none of the deficient chromosomes hybridize with the cDm 356 sequence, which is specific for chromosomal tips in *Drosophila melanogaster* (4). Therefore, we suggest that these deficiencies extend to the very end of the chromosome arm and represent one-break aberrations. There are two major problems involved in proving this supposition. First, there is presently no convenient assay for a telomere. All of the assays used here test for the presence of a marker (genetic, cytological, or molecular) that lies near or within the telomere region but not necessarily at the chromosome tip. Therefore, the loss of a distal marker does not prove that the entire telomere region is also missing. Second, the general approach taken here is a negative one. We have been unable to find evidence that the original telomeric region is present on the deficiency chromosomes. This is not evidence that this region is completely absent. It is not possible at this point to examine any one *y* mutation and say unequivocally that it is a terminal deficiency (1, 21), although the accumulated evidence from many independent mutations considerably strengthens the argument. In addition, the frequency of these deletions increases linearly with x-ray dose, further supporting the idea that they are one-break rearrangements.

Table 6. Mapping of *mu-2*

Chromosome constitution	Progeny		
	+	*y*	% *y*
X/X	33,396	3	0.009
II/II	28,241	2	0.007
III/III	10,561	13	0.12
III/TM3	17,044	1	0.006

It is obvious, however, that *mu-2* is responsible for the recovery of a class of x-ray-induced mutations that has rarely, if ever, been recovered before. After irradiating mature sperm with 4000 R of x-rays, Roberts (21, 28) found little evidence that any of the 31 newly-induced *y* mutations were associated with a terminal deficiency. He was able to find only one chromosome (out of more than 93,000) that appeared to carry a deficiency for the X chromosome tip. Our procedure of scoring for *y* deletions is similar to that used by Roberts, except that in the present case females were treated with 500 R. The frequency of deletions recovered from treated *mu-2* females is 1–3 × 10^{-3}, about 3-fold higher than that found by Roberts (28); but virtually all of these appear to be associated with terminal deficencies. Thus, the frequency of apparent terminal deficiencies from *mu-2* females is 100 times higher than that found by Roberts (21), while the x-ray dose is lower by a factor of 8.

The evidence presented here strongly suggests that the majority of mutations recovered among the progeny of *mu-2* females are terminal deletions. Assuming this to be the case, one is faced with the same problem that faced Muller (1–3)—i.e, how does one explain the massive amount of data showing that terminal deletions and inversions (one-break aberrations) are extremely rare compared with more complex, multiple-break rearrangements? In addition, telomeres may be necessary for the proper replication of linear DNA molecules (29). There seems to be no alternative to accepting the requirement for telomeres on linear chromosomes. How, then, does one account for the terminal deficiencies recovered in this study? They could be ring chromosomes, since rings are stable in *Drosophila* without the need for a telomere. However, cytological analysis shows all of the deficient chromosomes to be linear. Therefore, the broken ends must have been healed (18–20); in other words, new telomeres must have been produced *de novo*, or preexisting telomere sequences must have been added to the broken ends. There is molecular evidence to indicate that healing of linear DNA molecules in fact can take place. During macronuclear development in the ciliated protozoans *Oxytricha nova* and *Tetrahymena thermophila*, satellite-like sequences with telomeric properties are added onto the ends of linear subchromosomal DNA fragments (30–32). Similar mechanisms of chromosome healing might exist in *Drosophila*.

If *mu-2* is responsible for the recovery of stable terminal deficiencies, this has important implications in at least two areas, the genetic control of DNA repair and telomere structure. The present data suggest that *mu-2* is defective in repairing an x-ray-induced lesion, resulting in the loss of material distal to the lesion. The simplest hypothesis is that *mu-2* interferes with the repair of double-strand breaks. This might interfere with restitution as well as the bridge–breakage–fusion cycle, leaving unrepaired breaks open for a relatively long period. However, the broken chromosomes would not be recovered as stable deficiencies in such a repair–defective background unless the break is "healed." We suggest that the healing process is normally available in *mu*$^+$, but that the "normal" repair process is much more efficacious and handles the vast majority of breaks. It is only when the primary repair process is blocked that the effects of the secondary healing process can be seen. Similarly, in maize endosperm, breaks are repaired by joining the two broken ends

of sister chromatids, leading to a bridge–breakage–fusion cycle. In the embryo, the breaks are allowed to heal (19, 20), giving rise to stable terminal deficiencies.

In Muller's (2, 3) terms, the healing of a broken chromosome end requires the production of a new telomere, since the healed chromosome has lost its previous telomere. The nature of telomere synthesis is, of course, unclear because the molecular structure of the new telomeres has not yet been determined. However, given the high frequency with which the newly broken chromosomes can be recovered, it should be possible to recover new breaks within a limited region (say between *y* and *ac*) and, by using recombinant DNA techniques and linear plasmid vectors, to clone and molecularly characterize these new telomeres. It will then be possible to ask questions concerning the nature of the healing process and the sequences that can act as the DNA component of telomeres in *Drosophila*.

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