Genetic instability in Drosophila melanogaster: Cytogenetic analysis of MR-induced X-chromosome deficiencies
(chromosome breakage/hybrid dysgenesis/P elements)

M. M. Green*, Masa-Toshi Yamamoto†, and George L. Gabor Miklos‡§

*Department of Genetics, University of California, Davis, CA 95616; †Department of Cell Genetics, National Institute of Genetics, Mishima, Shizuoka 411 Japan; and ‡Developmental Neurobiology Group, Research School of Biological Sciences, The Australian National University, GPO Box 475, Canberra, A.C.T. 2601 Australia

Contributed by M. M. Green, March 10, 1987

ABSTRACT We present data that demonstrate that three MR elements isolated from wild populations of Drosophila melanogaster on two continents can cause large deletions of the X chromosome in males. The deleted chromosomes, termed mini-X chromosomes, are induced at a frequency of ≈1:4000 in chromosomes that are initially free of P elements. In situ hybridizations using a cloned P sequence as a probe fail to reveal any sequences homologous to the nomadic P family at the deletion breakpoints. Genetic analysis of 12 such mini-X chromosomes also reveals that there are no “hotspots” of chromosome breakage and that there must have been a minimum of three distinct distal breakpoints and five different proximal breakpoints in the formation of these deleted chromosomes. In fact all 12 proximal and 12 distal breakpoints may well be unique. Our data show that MR elements generate essentially random breaks along the X chromosome. We emphasize that we find no involvement of P sequences in the chromosome breakage process, consonant with the notion that MR elements exert their influence on processes involved in mitotic crossing-over.

Two major properties have been assigned to MR (mutation-recombination) elements that are widespread in natural populations (1–3). These elements generate elevated levels of mitotic crossing-over in premeiotic germ cells of both males and females (4–6) and also act as mutators by mostly facilitating the integration of P elements at various loci (7, 8). It is also known that two MR-bearing chromosomes inherited patrilocously are no more potent than a single one in terms of mutator activity (9). Furthermore, mapping of two second chromosome MR elements from sources as diverse as Haifa, Israel and Texas reveals very similar chromosomal locations in a short interval of ≈1 map unit (10, 11). In addition, cytogenetic analyses have revealed that MR elements can generate deletions of up to several contiguous gene loci in the regions including the loci of lethal giant larva (12), net (12), cinnabar (12, 13), black (12), purple (12), light (6), scarlet, and peach (M.M.G., unpublished data). They can also generate translocations and duplications (13). In some cases tandem duplications of at least four polytene chromosome divisions have been induced by MR on an X chromosome initially free of P elements (M.M.G., unpublished data). MR elements thus provide a genetic basis for the phenomena associated with the P-M system of hybrid dysgenesis (8, 14–16). It has also been claimed that MR factors and P factors are indistinguishable (16), and some studies have been predicated on this assumption (6).

We now report that MR elements can regularly produce massive deletions of most of the euchromatic part of the X chromosome at comparatively high frequencies in X chromosomes that are originally free of P elements. The deletion events, of the order of 15 million base pairs, are detected through the recovery of free X-chromosome duplications that retain the centromere and telomere but have most of the interstitial euchromatin deleted. These duplications were subjected to cytogenetic and in situ hybridization analyses, and the details of this study and a comparison of the MR and P-M systems are the subjects of this report.

MATERIALS AND METHODS

The relevant gene mutations employed in this study are given in Table 1. The nomenclature and characteristics of the complementation groups at the base of the X chromosome have been described (17, 18). The MR chromosomes used to generate rearrangements are denoted T007, 102, and h12 and were isolated from natural populations in Texas, California, and Israel. Flies were cultured on a conventional cornmeal/sucrose/brewer’s yeast medium at a room temperature of 22–25°C.

Polytene Chromosome Squashes and Biotinylated Probe Synthesis. Salivary gland chromosome preparations were made in 45% (vol/vol) acetic acid and dehydrated in 98% (vol/vol) ethanol for 2–4 hr. The air-dried preparations were incubated in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) at 70°C for 30 min, and hybridizations were performed overnight at 37°C with biotinylated probes at a concentration of 1 μg/400 μl of hybridization solution. Biotinylated probes of p25.1 (19) were synthesized by a standard nick-translation method using biotin-11-dUTP (Bethesda Research Laboratories). The hybridization mix consisted of 50% (vol/vol) formamide, 4× SSC, 1× Denhardt’s solution, 10% (wt/vol) dextran sulfate, and sheared calf thymus DNA at 250 μg/ml. (1× Denhardt’s solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin.) Slides were washed twice in 2× SSC at 60°C and then in 1× SSC at room temperature. They were then treated with the DNA detection system (Bethesda Research Laboratories) using alkaline phosphatase to localize sites of hybridization. In situ hybridization was in some cases also monitored by using labeled cloned DNA from the white locus as an additional internal control in the hybridization reaction.

Mitotic Chromosome Preparations. These were carried out as described (20) with the following minor modifications. Third-instar larval brains were dissected in 0.7% NaCl and transferred for 5–7 min to a solution containing 0.1% sodium citrate and 0.005% colchicine. The tissue was then fixed in ethanol/glacial acetic acid/distilled water, 3:3:4 (vol/vol), and rapidly minced with forceps. The slide was briefly washed in a solution of ethanol/glacial acetic acid, 1:1 (vol/vol), and after the further addition of 1 drop of glacial acetic acid, was air-dried at room temperature. Chromo-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†To whom reprint requests should be addressed.
Table 1. Key to the X-chromosome genetic symbols used

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Location</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>y</td>
<td>1A8–1B1</td>
<td>Yellow body color</td>
</tr>
<tr>
<td>su(s)</td>
<td>1B10–1C1</td>
<td>Suppresses sable body color</td>
</tr>
<tr>
<td>su(w*)</td>
<td>1E1</td>
<td>Suppresses white apricot eye color</td>
</tr>
<tr>
<td>w</td>
<td>3C2</td>
<td>White eye color</td>
</tr>
<tr>
<td>cv</td>
<td>5B</td>
<td>Crossveins absent</td>
</tr>
<tr>
<td>v</td>
<td>10A1</td>
<td>Vermillion eye color</td>
</tr>
<tr>
<td>f</td>
<td>15F1,2</td>
<td>Forked bristles</td>
</tr>
<tr>
<td>car</td>
<td>18D1,2</td>
<td>Carnation eye color</td>
</tr>
<tr>
<td>mal</td>
<td>19D</td>
<td>Maroon-like eye color</td>
</tr>
<tr>
<td>shak</td>
<td>19E2</td>
<td>Shaking under ether anaesthesia</td>
</tr>
<tr>
<td>R-9-28</td>
<td>19E3</td>
<td>Lethal complementation group</td>
</tr>
<tr>
<td>unc</td>
<td>19E8</td>
<td>Lethal complementation group, survivors have uncoordinated leg movements</td>
</tr>
<tr>
<td>fl</td>
<td>19F1</td>
<td>Lethal complementation group, survivors are of small size</td>
</tr>
<tr>
<td>eo</td>
<td>20A1,2</td>
<td>Duplicated or deleted external organs</td>
</tr>
<tr>
<td>wap</td>
<td>20A3</td>
<td>Lethal complementation group, survivors have wings set slightly apart</td>
</tr>
<tr>
<td>su(f)</td>
<td>20F</td>
<td>Suppresses forked bristles</td>
</tr>
</tbody>
</table>

somes were stained with Giemsa (Merck) in 0.01 M sodium phosphate buffer at pH 6.8.

RESULTS

Large X chromosome deletions generated by MR elements were recovered in a special crossing program in which males harboring a second chromosome MR element and an X chromosome with the recessive mutations scute and zeste were crossed to homozygous females possessing a compound double X chromosome [denoted C(1)DX] that carried the y, w, and f mutations (21). In three experiments in which males had one of three independently isolated MR elements, a total of 107,310 female progeny were scored. Among these female progeny, 25 were of the phenotype y⁺ w f, and 40 were of the phenotype y⁺ w⁺ f. It should be noted that exceptional females were produced in approximately equal frequencies by each of the three different MR elements. These results indicate that the y⁺ w f females were likely to carry not only the compound double X chromosome with its three mutant markers y, w, and f, but, in addition, have an X chromosome segment marked with y⁺ that is paternally derived. Similarly, the y⁺ w⁺ females are assumed to carry a paternally derived chromosome segment marked with y⁺ and w⁺. However, since these latter females were only poorly fertile, they were not investigated further. On the other hand, the y⁺ w f females were generally of good fertility, and cytogenetic studies were made to determine their precise constitutions.

Since MR elements are implicated in the generation of mitotic exchanges, it is possible that, in males harboring MR elements, single exchanges between the tip of an X chromosome (carrying the y⁺ gene) and a Y chromosome produce X–Y translocations, a viable product of which is recovered as a phenotypic y⁺ w f female. When such a female is crossed to a normal male, the males receiving the y⁺-bearing Y fragment will be sterile owing to the Y chromosome being incomplete.

Alternatively, if the paternal X chromosome undergoes breakage events at its distal and proximal ends and ligation involves the centromere-bearing proximal fragment with the telomere-bearing distal fragment, then a mini-X chromosome would result that would be deleted for most of the interstitial X euchromatin. Such a y⁺-bearing mini-X chromosome when present in the sperm fertilizing a compound double X, y w f-containing ovum will yield a zygote that develops into a y⁺ w f female. When such a female is bred to a normal male, her sons receive only the mini-X chromosome from the mother and so are sterile because of the complete absence of a Y chromosome.

To distinguish between these two modes of origin for the y⁺-bearing chromosome, namely X–Y exchanges or internal deletion of the X chromosome itself, true breeding stocks were established from individual y⁺ w f females. The primary nondisjunction in such females facilitates the recovery of y⁺ w f daughters. These when crossed to attached XY males lacking a free Y chromosome enable the construction of stocks in which both fertile sexes carry y⁺-bearing chromosomes.

Utilizing this genetic scheme, stocks of 12 independently derived y⁺-bearing chromosomes generated by one or other of three different MR elements were established to determine their mode of origin. If X–Y exchanges occur, the y⁺-bearing chromosomes will carry genes from the distal X-chromosome tip but none from the proximal euchromatin of the X chromosome. If, on the other hand, internal X-chromosome deletions occur, then the y⁺-bearing chromosomes should have genes from both the distal and proximal euchromatic regions.

Table 2 shows the results of the genetic tests of the 12 y⁺-bearing chromosomes. The results are unambiguous: all chromosomes carry some genes from polytene chromosome division 1 at the tip of the X chromosome and some genes from divisions 18–20 at the proximal end. These chromosomes thus originated from massive internal deletions of the X euchromatin followed by tip–base rejoicing. Mitotic cytology of representative y⁺ chromosomes confirms this conclusion (Fig. 1 a and b). The deleted X chromosomes revealed in these figures consist largely of the basal heterochromatic region of the X chromosome and are in essence mini-X chromosomes. As expected, in both mini-2 (Fig. 1a) and mini-77 (Fig. 1b), the heterochromatins of the mini-X chromosome is indistinguishable in length from that of the normal X chromosome in the same cell. The nucleolus organizer is seen as a clear constriction in the middle of the heterochromatin, and the four classical heterochromatic blocks are also clearly identifiable.

The data of Table 2 reveal a second important point relevant to the origin of the mini-X chromosomes. Their genotypes show that there is neither a common distal nor a common proximal point of chromosome breakage. At the distal end, minimally three different sites of chromosome breakage must have occurred, and there must have been at least three distinct sites of proximal breakage. This point will be considered in more detail below.

In an attempt to gain further insight into the mechanism of mini-X chromosome formation, we determined whether an MR element could cause deletions in a ring-X chromosome.

Table 2. Approximate genetic constitution of the 12 y⁺-bearing mini-X chromosomes derived from the crossing program MRΔ δ × C(1)DX, y w f ∃ ?

<table>
<thead>
<tr>
<th>Mutation/location</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>y/1A8–1B1</td>
<td>1</td>
</tr>
<tr>
<td>su(s)/1B10–1C1</td>
<td>2</td>
</tr>
<tr>
<td>su(w*)/1E1</td>
<td>3</td>
</tr>
<tr>
<td>car/18D1,2</td>
<td>4</td>
</tr>
<tr>
<td>mal/19D</td>
<td>5</td>
</tr>
<tr>
<td>su(f)/20F</td>
<td></td>
</tr>
</tbody>
</table>

Number recovered 3 1 1 6 1

Polytene chromosome division 1 is at the tip of the X chromosome while divisions 18–20 constitute the proximal euchromatin. + Presence of the wild-type allele of the gene on the mini-X; −, its absence. The number of y⁺-bearing chromosomes recovered is shown.
The rationale for this experiment stems from the following considerations. In a normal, replicated rod X chromosome, the reunion of the one break in the proximal euchromatin of one chromatid with another break in the distal tip euchromatin of its sister chromatid leads to a mini-X chromosome and an almost fully duplicated chromosome. The duplication-bearing zygotes will be inviable. In a replicated ring chromosome, however, if the two breaks occur just as above in sister chromatids, then a dicentric double-sized ring is formed, which is not recoverable. Thus it is not possible to generate a mini-ring chromosome from a full-sized ring by two-break proximal-distal sister chromatid exchanges. However, if the breaks occur in the proximal and distal euchromatin of the same chromatid (isochromatid breaks) or as chromosome breaks, then subsequent segregation can generate a mini-ring chromosome.

Accordingly experimental males were constructed that carried an MR element and a ring X chromosome (denoted Xc2) carrying the markers cv vf. These were crossed to compound double X, y w f females, and 29,391 female progeny were scored for the presence of females of the genotype y' w f. Two such individuals were found but only one proved to be fertile. A balanced stock was constructed, and the y'-bearing chromosome was subjected to genetic analysis. It was found to carry the distal y' gene and the very proximal su(f) gene and was therefore equivalent to a mini-X chromosome. Mitotic chromosome cytology clearly shows this chromosome to be a mini-ring X chromosome (Fig. 1c) whose most likely origin was by isochromatid or chromosome breakage. The finding that the frequency of mini-X chromosomes induced in rod X chromosomes by MR elements is at a frequency of $\text{=1:4000}$, whereas the frequency of mini-X chromosomes from a ring X is $\text{=1:15,000}$, suggests, but does not prove, that sister chromatid exchanges do occur. The implications of this would be that since the reunion products of proximal-distal sister chromatid exchanges in a ring X chromosome are not recoverable, their loss would result in a lower frequency of recoverable mini-X chromosomes from a ring chromosome.

We noted earlier that among the five classes of mini-X chromosomes produced under the aegis of MR activity, there must have been at least three distinct distal sites of breakage and at least three proximal sites of breakage. To determine whether the number of sites was even higher than this figure, we undertook a detailed mapping of four mini-X chromosomes within class 4 (Table 2), whose proximal breakpoints occurred between the loci of mal* and su(f)*. This genetic interval has been virtually genetically saturated and contains at least 27 lethal and visible complementation groups and $>100$ chromosomal rearrangements (17, 18). Utilizing much of this wealth of genetic information, we determined the genetic breakpoints of the four mini-X chromosomes (Fig. 2). Chromosomes mini-2 and mini-ring break between the loci of shak* and R-9-28*. Chromosome mini-77 is broken between unc* and if1*, and mini-6 is broken between eo* and wap*.

Thus among four mini-X chromosomes of the one class, three different genetic breakpoints occurred in the proximal region. It may well be that each mini-X chromosome of this class not only has its own distal and proximal breakage sites, but that all 12 mini-X chromosomes are unique. Finally, it should be noted that the fine level of mapping achieved in this genetic interval cannot be realized cytologically. Had $P$ elements been present at the sites of chromosome breakage, then there must have been multiple sites of insertion within short genetic intervals at both the tip and the base of the X chromosome. 

**MR Activity and P Element Transposition.** Since $P$ element transposition is influenced by the presence of MR elements and since $P$ elements have already been associated with the induction of mini-X chromosomes and specific X chromosome inversions (22-24), we performed in situ hybridization experiments with a $P$-element probe to determine whether $P$ elements are implicated in the genesis of the mini-X chro-
Fig. 2. The genetic content of the four mini-X chromosomes utilized in these studies. The available lethal and visible complementation groups in the proximal region of the X chromosome are as shown, as well as their approximate cytological locations. The tested lethal and visible mutations that are covered by the mini-X chromosomes are denoted (+); those that remain uncovered by the mini-X chromosomes are shown (−). Relevant genetic symbols are explained in Table 1 and in refs. 17 and 18. It should be noted that the mini-2 chromosome has now been found to extend further distally than reported earlier (21).

One possibility is that P elements become integrated both proximally and distally into an initially P-free X chromosome and should they synapse, a loop configuration for the intervening stretch of chromosome could ensue. Subsequently, an exchange within the paired P sequences would generate a centric mini-X chromosome and an acentric chromosome whose length corresponds to the interval between the P elements. It should again be stressed that based on the partial genetic mapping of the mini-X chromosomes given in Table 2 and Fig. 2, it is clear that any mechanism that involves P element synopsis requires P sequences to occupy a minimum of three different distal X chromosome sites and at least five proximal sites.

In situ hybridization was carried out on polytene chromosomes of three stocks containing the chromosome mini-2, mini-77, or mini-ring by using a cloned genomic DNA fragment from region 17C containing a complete P element (p25.1) as a probe (19). Chromosome mini-6, which breaks in the cytologically difficult region 20, proved to be refractory to consistent cytological analysis and was thus excluded from the in situ experiments. The in situ hybridization results for chromosome mini-2 are shown in Fig. 1d. P-related sequences are found near bands 1B9-10 but do not occur at the 2C-19E breakpoint that characterizes this mini chromosome. As expected, hybridization also occurs at region 17C on the intact X chromosome. These internal controls on both the normal X chromosome as well as the mini-X chromosome mean that if P-related sequences are present at the breakpoint then they are too small to be detected by this procedure. Similar results were obtained with the chromosomes mini-77 and mini-ring. With mini-77 the only site of hybridization was at 17C, whereas with the mini-ring there was hybridization at 17C, 68F, 86E, and 86F, 87C, 87D, and 89C. In no case was there any hybridization to a region that spans the site of the proximal–distal breakage–reunion event.

**DISCUSSION**

The regular induction of mini-X chromosomes documented here can be considered as an extension of the germ-line mitotic exchange processes associated with the presence of MR elements in a genome. It is, however, necessary to invoke chromosome (or chromatid) breaks to produce a mini-X chromosome. Mini-X chromosomes have been made (22), but these experimental X chromosomes already contained a number of P sequences prior to the induction of rearrangements by the P-M system. It was found that breakage hotspots preferentially occurred at or near preexisting P-sequence sites (23) and that the majority of the induced chromosomal rearrangements carried P sequences at or near one or both breakage–reunion sites.

In our studies, however, the incoming experimental X chromosome was free of P elements, and we found no evidence for any P sequences at the sites of chromosome breakage of the rearranged chromosomes. Furthermore our in situ hybridization assay did detect P sequences elsewhere in the genome. One would need to argue that P sequences are indeed still present at the different breakage sites of the various mini-X chromosomes, but all such sites are located in underreplicated regions of the polytene X chromosome and so do not provide a sufficiently amplified target for the incoming probe. However, our data are paralleled by those of an independent study (B. Mechler, personal communication), in which much smaller MR-induced deficiencies were made at the lethal giant larva locus. All three deficiencies examined so far by direct DNA sequencing through breakpoints have proved to be free of P (or any other) nomadic sequences.

However, it could still be imagined that P sequences were mobilized into the proximal and distal regions of the P-free X chromosome, and, if synopsis of such sequences occurred, exchange events would need to have been such as to excise both P elements in each instance.

What is still not clear, however, are the mechanisms by which the presence of MR elements in a genome leads to chromosome breakage that culminates in mitotic exchanges between homologs, or within chromosomes to yield mini-X chromosomes. Since MR elements are intimately associated with the transposition of nomadic P elements, it seemed reasonable to assume that P elements were involved in breakage processes. In retrospect, however, it appears unlikely that P elements generate rearrangements when the chromosome is initially free of P elements.

Our results are thus consistent with the notion that MR elements are causal agents of chromosome breakage, a consequence of which is the potential for genomic reorganization. This breakage, however, is neither associated with nor predicated upon the presence of P elements at or near breakage–reunion sites. Thus some important aspects of both the genetic and molecular bases of P-M-induced hybrid dysgenesis, as well as the relationship between MR-bearing chromosomes and P factor-bearing chromosomes require clarification.
Bregliano and Kidwell (16) have claimed that “the chromosomal component of the \( P-M \) interaction consists of one or more \( P \) factors” and that “the available evidence overwhelmingly supports the interpretation that \( MR \) factors may be indistinguishable from \( P \) factors.” It has also been explicitly argued that the syndrome of hybrid dysgenesis is largely due to the activation of members of the mobile \( P \) element family that is present in \( P \) strains but absent from \( M \) strains. The “refined” version of this \( P \)-factor hypothesis of Bingham et al. (15) accounts for apparent oddities such as \( Q \) strains (in which hybrid dysogenesis is not observed in appropriate crosses) by postulating that \( Q \) strains “do not have copies of the \( P \) element that possesses the functional genetic elements which cause hybrid sterility, but do have \( P \) elements with all other properties attributable to the aggregate of \( P \) elements in \( P \) strains” (15).

In the light of these hypotheses and the available data, how then do the \( MR \) and \( P-M \) systems interrelate? Is an \( MR \) element a single complete \( P \) element? It is already known that a singed mutation caused by an insertion of a complete \( P \) element can induce the reversion of a raspberry eye color mutation that is itself due to the insertion of an incomplete \( P \) element (8). However, the same singed mutation with its resident complete \( P \) element is incapable of generating mitotic recombination (8). Thus a single complete \( P \) element and an \( MR \) element are not identical. Furthermore, of six \( MR \) elements tested for ovarian dysgenesis, only three produced substantial breakdown (10, 13). In addition, ovarian dysgenesis and \( MR \) activity can be uncoupled by crossing-over (8, 10). Hence there is no direct connection between \( MR \) activity and gonadal breakdown.

It is also clear that the site specificity originally seen in certain rearrangements induced under the \( P-M \) system was a consequence of \( P \) elements that already existed in the chromosome being tested (22–24). If one begins with an X chromosome that already contains \( P \) elements and puts it through the \( P-M \) “mill,” then the “hotspots” of breakage-reunion are found at or near \( P \) element sites. If, however, the incoming chromosome is free of \( P \) elements, such preexisting constraints do not apply, and one deals essentially with random chromosome breakage events.

In our view, while \( MR \) and \( P-M \) elements are part and parcel of the same mutator system, it is premature to conclude that an \( MR \) element and a \( P \) factor are indistinguishable. It is also clear that some of the difficulties of interpretation of phenomena in the \( P-M \) system, such as cytotype, are presently more easily conceived of in terms of the inheritance of \( MR \) elements than of \( P \) factors (10).

We gratefully acknowledge the advice of Professors G. Lefevre, A. Schalet, J. Eeken, and T. Homyk concerning fly stocks. We also thank Marilyn Miklos for the preparation of the manuscript.