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Genetic–molecular basis for a simple *Drosophila melanogaster* somatic system that detects environmental mutagens

(mutagen testing/somatic mutation/deletion induction)

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ABSTRACT **We have developed a simple, objectively scorable test for the mutagenicity of chemical compounds which can be fed *Drosophila melanogaster*. The test depends upon the somatic reversion of the X chromosome, recessive eye color mutation, white-ivory (w^i) to wild type (w^+). Reversions are scored as clones of w^+ facets in the w^i eyes of eclosing adults. To increase the sensitivity, a tandem quadruplication containing four w^i mutations was synthesized. Thus, in homozygous females eight w^i mutations are potentially revertible. Six mutagenic compounds, all alkylating agents, all gave positive results at several concentrations tested. Molecular analysis demonstrates that the induced reversions, germinal and somatic, are associated with the loss of 2.9-kilobase DNA duplicated in the w^i mutation.**

The need for a simple, utilitarian genetic test for environmental mutagenic compounds was dramatically demonstrated by the finding of McCann *et al.* (1), employing the Ames (2) *Salmonella* test, that most carcinogenic compounds are also mutagenic. The elegance and utility of the Ames test and its more recent improvement (3) cannot be denied. Nonetheless, there are cogent reasons for supplementing or complementing the Ames test with a comparable eukaryote test system. There are fundamental differences in how the genomic DNA of prokaryotes and eukaryotes is organized, which could conceivably influence the outcome of the Ames test. Similarly, there are real differences in prokaryote and eukaryote metabolism that do affect the outcome of the Ames test—e.g., compounds that in eukaryotes are metabolically converted to mutagens are not so converted in prokaryotes. On hand are a number of genetic and cytological tests for mutagens that employ intact eukaryote organisms or cultured cells. There is no need to review these test systems except to note that each suffers from one or another limitation. By and large, most tests are not simple to execute or the nature of the genetic damage is unclear. Historically, the eukaryote organism and mutagen test of choice have been *Drosophila melanogaster* and X chromosome lethal mutations—the *C1B* test and later improvements. Yet, even this comparatively simple test is wanting, because when the results are positive the precise nature of the induced lethal mutations is unclear. In fact, a review of the pertinent *D. melanogaster* mutagen test literature (4) demonstrates that in general such tests, whether somatic or germinal, are ambiguous when the issue of the nature of the mutagen-induced genetic lesion is raised.

We, therefore, undertook the development of a *D. melanogaster* somatic mutagen test based on the reversion of the X-linked eye color mutation white-ivory (w^i) to wild type (w^+). Our choice of w^i stems from two observations. First, it has been known for more than 25 years that w^i reverts to w^+ both somatically and germinally, spontaneously and after x irradiation (5–7). In the case of somatic reversions, these are readily scored as spots or clones of red, probably w^+, eye facets on a background of ivory, w^i, facets. The frequency of both somatic and germinal reversions increases after x-irradiation of larvae or adults, respectively. Second, the nature of the w^i mutant and its germinal w^+ reversions have been defined molecularly: w^i is a 2.9-kilobase (kb) tandem duplication of w^+ DNA appended directly onto a normal w^+ gene (8). Reversion of w^i to w^+, spontaneous and x-ray-induced, is associated with the more or less clean loss of the appended 2.9 kb, thereby restoring an intact w^+ gene. Thus, any agent, physical or chemical, that increases the reversion frequency of w^i to w^+ does so presumably by deleting the appended 2.9 kb and is, therefore, a deletion-making agent.

Although in homozygous females and males the somatic and germinal reversion frequency of w^i, spontaneous or x-ray-induced, is high compared to the vast majority of spontaneous *D. melanogaster* mutations, it is too low to be used as a basis for a routine test for mutagenic compounds. If, however, the number of copies of the w^i mutation in the fly's genome can be increased, it follows that the number of "targets" for reversion to w^+ also increases. An increased "target" number translates into an increased likelihood of reversion and concomitantly into an increased sensitivity to environmental mutagens capable of generating DNA deletions. In the narrative that follows we will document the synthesis of a tandem quadruplication of the w^i mutation. We will demonstrate that when chemical mutagens are fed to quadruplication larvae the frequency of somatic reversions of w^i to w^+ is increased significantly. Finally, we will establish at the molecular level that chemical-mutagen-induced reversions of w^i result from the deletion of the appended 2.9 kb.

MATERIALS AND METHODS

Synthesis of a Tandem Quadruplication of w^i [*Dp(1:1:1:1)-w^i*]. The w^i mutation is X chromosome linked, mapping to position 1.5. The synthesis of the quadruplication was envisioned to be a two-step process. In the first step a tandem duplication of w^i [*Dp(1:1)w^i*] would be generated by x-irradiating homozygous w^i females. Subsequently, unequal crossing-over in homozygous *Dp(1:1)w^i* females would be exploited to generate a tandem w^i triplication [*Dp(1:1:1)w^i*] and in homozygous triplication females to generate a tandem w^i quadruplication [*Dp(1:1:1:1)w^i*]. The synthesis of *Dp(1:1)w^i* took advantage of the fact that tandem duplications of the *w* locus can be objectively identified phenotypically (9). Thus, females possessing one wild-type X chromosome and one bearing a tandem duplication of the X chromosome recessive eye color mutation zeste [*Dp(1:1)z*] are wild type in eye color, but females possessing one X chromosome bearing

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Abbreviations: kb, kilobase(s); EMS, ethyl methanesulfonate; ENU, ethyl nitrosourea; DEB, diepoxybutane; TEM, triethylene melamine.

a tandem duplication of the w^+ locus and the homologous X bearing *Dp(1:1)z* exhibit a variegated zeste eye color phenotype.

In practice, homozygous y^2w^i females (y^2 = yellow-2 body color at position 0.0 on the X chromosome) were irradiated with ≈4000 rads (1 rad = 0.01 gray) of x rays and crossed to *Dp(1:1)z* males. In each of two independent experiments a single female was found of the zeste-variegated eye color. Retesting the presumptive *Dp(1:1)w*^{i} chromosomes with *Dp(1:1)z* confirmed that each generated the zeste-variegated eye color. In addition, the eye color of *Dp(1:1)w*^{i} males is distinctly darker than that of w^i males, and similarly the eye color of homozygous *Dp(1:1)w*^{i} females is distinctly darker than that of homozygous w^i females. These eye color phenotypes are consistent with increased dosage of the w^i mutation. Finally, salivary gland polytene chromosome cytology confirmed each *Dp(1:1)w*^{i} to be a tandem duplication involving a short interval of the X chromosome extending from section 3A to section 3C, thereby including w^i.

To synthesize w^i triplications, homozygous y^2 *Dp(1:1)w*^{i} females were crossed to *Dp(1:1)z* males and female progeny near zeste in phenotype were sought. Three independent *Dp(1:1:1)w*^{i} chromosomes were recovered. As expected, the eye color of *Dp(1:1:1)w*^{i} males is darker than that of *Dp(1:1)w*^{i} males and the eye color of homozygous *Dp(1:1:1)w*^{i} females is darker than that of homozygous *Dp(1:1)w*^{i} females.

The synthesis of w^i quadruplications entailed crossing homozygous y^2 *Dp(1:1:1)w*^{i} females to *Dp(1:1)z* males and recovering female progeny with a zeste eye color. Two independent *Dp(1:1:1:1)w*^{i} chromosomes were recovered. As expected, the eye color of *Dp(1:1:1:1)w*^{i} males is darker than that of *Dp(1:1:1)* males and that of homozygous *Dp(1:1:1:1)w*^{i} females is darker than that of homozygous *Dp(1:1:1)w*^{i} females. We established by Southern blot hybridization (data not shown) that in the series w^i, *Dp(1:1)w*^{i}, *Dp(1:1:1)w*^{i}, and *Dp(1:1:1:1)w*^{i}, paralleling the increase in eye color, there is a quantitative increase in white locus DNA with each increase in the number of w^i mutations. Synthesis of greater multiples of w^i was stopped at this point because homozygous *Dp(1:1:1:1)w*^{i} females are sterile.

The eye color of homozygous *Dp(1:1:1:1)w*^{i} females is so dark as to make objective scoring of somatic reversion of w^i difficult if not impossible. To remedy this situation, homozygous *Dp(1:1:1:1)w*^{i} females were made homozygous for the third chromosome eye color mutant scarlet (*st*). By incorporating *st* into the genotype, the biosynthesis of brown eye pigment is blocked. Thus the eye color phenotype of flies homozygous *Dp(1:1:1:1)w*^{i} and *st* is a pale yellow and somatic reversions of w^i to w^+ should be scorable as a clone of red facets on the pale yellow background. Pilot experiments in which somatic reversions of w^i to w^+ were sought by x-irradiating first-instar larvae of the genotype homozygous *Dp(1:1:1:1)w*^{i}; *st* confirmed that w^i reversions of any clone size can be objectively and easily scored in the eyes of eclosing adults.

For test purposes a constant breeding stock was synthesized of the genotype *Dp(1:1:1:1)w*^{i}*/FM6 l(1)66a; st/st* [*FM6* is a multiply inverted balancer X chromosome carrying the dominant *Bar* eye marker and a recessive lethal mutation, *l(1)66a*]. This stock is maintained by the cross *FM6/Dp-(1:1:1:1)w*^{i} females and *Dp(1:1:1:1)w*^{i} males; half the female progeny are homozygous *Dp(1:1:1:1)w*^{i}; all the males are *Dp(1:1:1:1)w*^{i}, since the *FM6* males die.

Mutagen Application. For somatic tests a number of chemical mutagens—ethyl methanesulfonate (EMS), ethyl nitrosourea (ENU), triethylene melamine (TEM), diepoxybutane (DEB), mitomycin C, and *cis*-diammine dichloroplatinum(II) (cisplatin)—were fed to first-instar larvae according to one of two schemes. Gravid females of the appropriate genotype were allowed to oviposit for 24 hr on standard *Drosophila* medium in either 190-ml milk bottles or 30-ml vials. Females were removed and eggs were allowed to hatch during the ensuing 24 hr. An aqueous solution of a chemical mutagen in appropriate concentration was then pipetted onto the surface of the medium, 1 ml to the bottles, 0.2 ml to the vials. Development proceeded at 25°C to eclosion of adults, which were then scored.

Where chemical-mutagen-induced germinal reversions were sought, males that had been fed the mutagen as larvae were crossed either to females homozygous for a small white locus deficiency (w^-) or to double X females [*C(1)DX*] homozygous for the recessive mutants yellow body (*y*) and forked bristles (*f*). In the first cross the female progeny were scored for reversions; in the second cross the male progeny were scored.

Nucleic Acid Procedures. We analyzed the molecular status of germinal reversions of w^i to w^+ induced by chemical mutagens as follows. High molecular weight DNA was prepared from adult flies as described in ref. 10. One-microgram samples of each DNA were digested with appropriate amounts of restriction enzymes under their optimal conditions. The digests were subjected to electrophoresis in Sigma type I agarose gels and then transferred to nitrocellulose filters as described in ref. 11. Hybridization was carried out with nick-translated (12) gel-purified fragments derived from a subclone of λm2.1 (16) as described in ref. 13.

Somatic reversions of w^i to w^+ were also analyzed molecularly. For this purpose first-instar w^i larvae were fed DEB. Those eclosing adults exhibiting a mosaic w^+ clone of minimum size, 15 facets, were collected and quick frozen. While frozen, heads (500–1000) were separated from bodies and their DNA was extracted and probed as outlined above.

RESULTS

Preliminary to our using chemical mutagens, we conducted two experiments designed to answer the question: Is the likelihood of reversion of w^i to w^+ increased appreciably in the quadruplication of w^i compared to a single w^i mutation? Since x rays are known to revert w^i to w^+, we first compared the frequency of germinal reversions of w^i induced in females of the genotype *Dp(1:1:1:1)w*^{i}*/w*$^-$ (w^- a small deletion) x-irradiated with 4000 rads and crossed to w^- males. Among the progeny 17 w^+ reversions were found among 12,226 *Dp(1:1:1:1)w*^{i}-bearing progeny scored. When clusters are scored as one reversion, the frequency of reversion becomes 11/12,226 or 0.9×10^{-3} per chromosome scored. This is nearly an order of magnitude greater than the frequency of reversion of 0.11×10^{-3} (10/88,000 progeny) found when homozygous w^i females were irradiated with the same x-ray dose (7).

In a second experiment we measured the frequency of somatic reversions of w^i when 72-hr-old *Dp(1:1:1:1)w*^{i} larvae are exposed to ≈1000 rads of x-rays. Somatic reversion frequency was estimated as the number of eclosing adults with w^+ eye clones per total number of adults scored. We found among homozygous *Dp(1:1:1:1)w*^{i} females a somatic reversion frequency of 188/519 or 36% and among *Dp(1:1:1:1)w*^{i} males a somatic reversion frequency of 86/500 or 17%. (Note the somatic reversion frequency in females with eight w^i genes is twice that of males with four w^i genes.) In equivalent experiments with ≈1000 rads of x rays, the frequency of w^+ eye mosaics in w^i/w^i females was 41/536 or 7.6% and in w^i males it was 29/524 or 5.5% (14). Taking these results at face value, it is clear that somatic reversion frequency is a function of the number of w^i genes in the fly's genome. In other experiments (data not given), we found the x-ray-induced somatic reversion frequency is linear as to number of w^i genes in the genome and linear as to x-ray dose

up to ≈1600 rads, the maximum dose tolerated for reasonable survival.

Table 1 is a compilation of the somatic reversion results obtained after testing the several chemical mutagens listed above. A number of points are apparent from the data presented. All the compounds tested increased the frequency of somatic reversion over that of the untreated controls for each concentration employed with both the w^i and the $Dp(1:1:1:1)w^i$ chromosomes. The $Dp(1:1:1:1)w^i$ chromosome, not unexpectedly, is more prone to somatic reversion than is the w^i chromosome. There is good reason to believe that in the case of TEM the way our experiment was carried out resulted in an underestimate of the somatic reversion frequency. Our cultures were seeded with live yeast, and the mutagenic potency of TEM is markedly reduced in the presence of live yeast (15). What is particularly striking is the increased proneness to somatic reversion induced by DEB in the $Dp(1:1:1:1)w^i$ chromosome as compared to a single w^i gene.

We compared the size of somatic clones in control and mutagen-treated $Dp(1:1:1:1)w^i$ flies. There is a distinct difference in clone size. In untreated controls, 75% (121/162 scored adults) of the reversions were limited to one or two facets; the remainder were all larger. Among TEM-treated flies 46/227 (20%) of the reversions included 1 or 2 facets; the remainder were larger. In contrast, among DEB-treated flies only 266/816 (32%) of the reversions included one or two facets; the remainder of the clones were larger. For cisplatin-, ENU-, and EMS-treated flies, for which the data are more limited, the respective frequencies of one- and two-facet reversions were 2/90 (2%), 69/122 (56%), and 38/66 (57%). Taking these data at face value, we emphasize two points: First, the chemical mutagens acted early in larval development to generate reversions. Second, if the small, one- or two-facet, clones, which are the most difficult to score are ignored, the difference between control and treated flies is accentuated.

Subsequent to treating larvae with mutagens for somatic reversions, we tested progeny of eclosing $Dp(1:1:1:1)w^i$ males, seeking germinal reversions of w^i. The results, tabulated in Table 2, are essentially self-explanatory. Reversions to w^+ were obtained with each mutagen. Those occurring in clusters are expected if the mutagen generates a reversion in premeiotic cells, albeit single reversion could also be premeiotic in origin.

Table 2. Frequencies of reversions to w^+ among progeny of $Dp(1:1:1:1)w^i$ males fed mutagenic agents as larvae

Mutagen	Conc., mM	Total w^+ progeny	Total progeny scored
None	—	0	20,441
ENU	1	23 (19 + 2 + 1 × 2)*	18,266
	2	18 (17 + 1)	12,268
TEM	0.1	16 (16)	13,466
DEB	47	1 (1)	15,112
	93	7 (7)	5,460

*Independent reversions of cluster size 19, 2, and 1 twice.

We undertook a molecular analysis of reversions generated by chemical mutagens to establish whether or not reversion of w^i to w^+ was associated with deletion of DNA. Fig. 1 is restriction maps of the w^+ gene from the Canton-S wild stock and of the w^i mutation plus the probe (double-headed arrow), an *Xba* I–*Sma* I derivative of the Canton-S w^+ gene employed in the Southern blot hybridization experiments. In Fig. 2 the results of the blot hybridization experiments are presented. These results (lanes 1–5) are essentially self-explanatory: w^i can be distinguished from w^+ and the mutagen-induced reversions all contain both w^+ and w^i DNA, demonstrating that the germinal reversions from w^i to w^+ were associated with the loss of the duplicate 2.9 kb of w^i.

Blot hybridization results concerned with the somatic reversions of w^i to w^+ are presented in lanes 6–10 of Fig. 2. In lane 6 a faint band of w^+ DNA can be seen, a band not present in lane 7. (We are unable to account for the additional faint band in lane 6.) The blot hybridization results after mixing a large amount of w^i DNA with a small amount of w^+ DNA in lane 9 demonstrate that relatively small amounts of somatic w^+ DNA can be detected in the presence of an excess

Table 1. Frequency of somatic reversions among eclosing adult flies fed chemical mutagens as larvae

Chemical mutagen	Conc., mM	Reversion frequency: Males w^i	Males $Dp(1:1:1:1)w^i$	Homozygous females w^i	Homozygous females $Dp(1:1:1:1)w^i$
None	—	0.09 (1/1169)	0.9 (57/6264)	0.18 (2/1137)	1.5 (31/2005)
	—	—	1.6 (32/2036)		1.2 (42/3477)
EMS	5	3.6 (25/686)	6.3 (17/271)	NT	NT
	10	4.1 (50/1220)	11.9 (33/277)	NT	NT
	20	5.8 (14/240)	13.4 (16/119)	NT	NT
ENU	0.5	1.9 (39/2090)	6.4 (45/699)	NT	NT
	1.0	5.3 (63/1194)	7.6 (32/420)	NT	NT
	2.0	6.8 (51/745)	12.6 (45/356)	NT	NT
TEM	0.05	NT	1.2 (15/1279)	NT	2.5 (15/611)
	0.1	0.18 (4/2172)	1.8 (19/1041)	0.7 (15/2058)	3.7 (24/648)
	0.2	0.31 (5/1625)	4.2 (35/839)	0.6 (10/1546)	4.7 (24/512)
	0.4	0.26 (7/2654)	5.1 (62/1217)	0.9 (29/2936)	13.0 (33/253)
DEB	12	NT	7.4 (56/753)	NT	16.7 (28/168)
	23	2.0 (47/2387)	10.6 (84/792)	3.1 (71/2300)	18.2 (42/231)
	47	2.7 (77/2852)	25.2 (229/910)	7.2 (196/2725)	41.7 (83/199)
	93	3.3 (72/2208)	39.8 (198/497)	6.5 (153/2361)	61.1 (66/108)
Mitomycin C	0.2	NT	1.0 (3/309)	NT	3.1 (12/388)
	0.5	NT	6.5 (8/123)	NT	10.3 (12/117)
	1.0	NT	15.4 (19/123)	NT	35.3 (49/139)
Cisplatin	0.2	NT	10.5 (48/459)	NT	22.9 (111/484)
	0.5	NT	18.9 (97/513)	NT	39.4 (282/716)

For each genotype the frequency of mosaic flies is given as percent followed by the number of mosaics/total flies scored. NT, not tested.

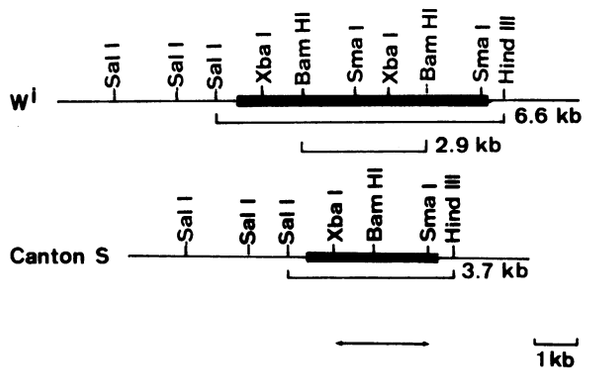

FIG. 1. Restriction maps of the w^+ (Canton-S wild type) and w^i genes (8). The two-headed arrow denotes the 2.1-kb *Xba* I–*Sal* I fragment included in λm2.1 used as a probe.

of w^i DNA. In Fig. 3 densitometer tracings of the w^i and w^+ blots in lanes 6 and 7 are given, confirming that a faint band of w^+ DNA occurs in the somatic reversions of w^i. Thus, the somatic reversions, like the germinal reversions of w^i, are associated with the deletion of the added 2.9 kb.

DISCUSSION

We believe the foregoing results form the basis for a simple, objective test for the mutagenicity of chemical compounds which can be fed to *Drosophila* larvae. Using the tandem quadruplication demonstrably sensitizes detection of somatic reversions of w^i to w^+. This increased sensitivity can be illustrated by taking the data in Table 1—e.g., the frequencies of DEB-induced somatic reversions—at face value. We calculate for 23 mM DEB that a total of 981 homozygous w^i female progeny must be scored to obtain a significant (at the 1% level) increase in reversion frequency in 99% of the trials. For the same DEB concentration only 133 homozygous $Dp(1:1:1:1)w^i$ females need be scored to achieve the same level of significance in the same number of trials. At 93 mM DEB, 418 homozygous w^i females and only 20 homozygous $Dp(1:1:1:1)w^i$ females need be scored for the same level of

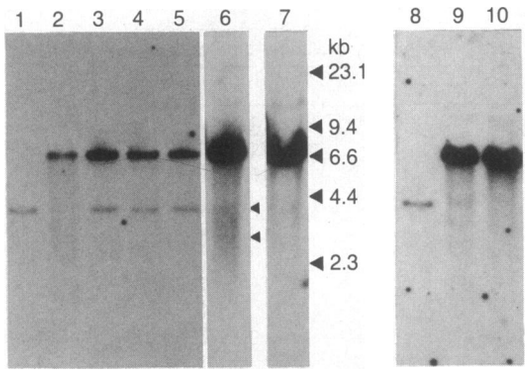

FIG. 2. Blot hybridizations of *Sal* I–*Hin*dIII digests of DNA from w^+ (Canton-S), w^i, $Dp(1:1:1:1)w^i$, germinal reversions induced in $Dp(1:1:1:1)w^i$, and somatic reversions induced in w^i. Restriction fragments were separated on 0.6% agarose gels, transferred to nitrocellulose, and hybridized with the ^{32}P-labeled *Xba* I–*Sma* I fragment of λm2.1. Lane 1 contains 0.8 μg of DNA from w^+ males, lane 2 contains 0.2 μg of DNA from $Dp(1:1:1:1)w^i$ males, and lanes 3, 4, and 5 contain 0.8 μg of DNA from revertants in $Dp(1:1:1:1)w^i$ induced by ENU, TEM, and DEB, respectively. Lane 6 contains 5 μg of DNA from heads with DEB-induced somatic reversions of w^i; lanes 7 and 10 contain 5 μg of DNA from heads of untreated w^i flies. Lane 8 contains 0.36 μg of DNA from heads of w^+ males and lane 9 contains a mixture of 0.12 μg of DNA from w^+ heads and 5 μg from untreated w^i heads.

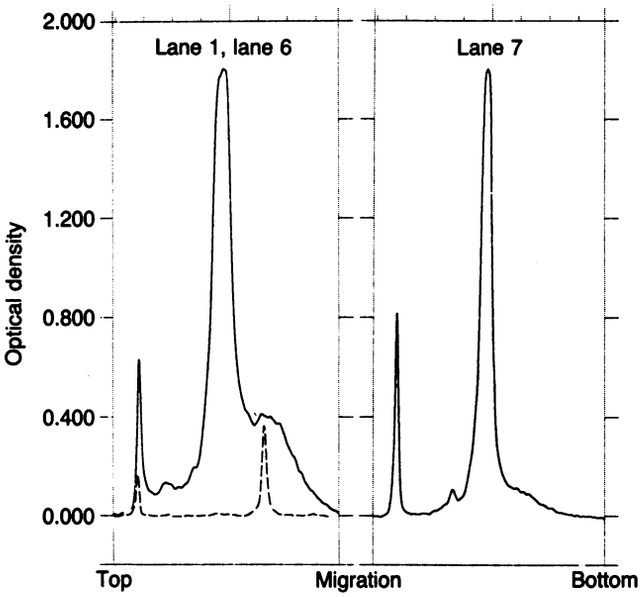

FIG. 3. Densitometer tracings of lanes 6 and 7 of Fig. 2 in the region of hybridization of w^+ DNA. Note the shoulder in 6 is absent from 7. Broken line is densitometer tracing of lane 1, Fig. 2.

significance. Therefore we believe that by employing the $Dp(1:1:1:1)w^i$ chromosome one component in a general test—namely, substantial sensitivity—can be achieved.

A second element in any general test is objectivity in scoring. Can somatic reversions be scored easily? To test objectivity in scoring somatic reversions of w^i, we enlisted the aid of two volunteers, a biophysicist and a pharmacologist, each without prior experience in handling *Drosophila*. Each volunteer was tested for ability to identify reversions of different clone size. Both subjects scored somatic clones accurately and correctly irrespective of clone size. We conclude that no special skills are needed to score somatic reversions of w^i.

Finally, a brief comment is in order on the scope of chemical mutagens detectable by the w^i system. On the basis of the earlier results with x-rays, we anticipated that mutagens such as DEB, TEM, and mitomycin C, known to produce genetic deletions in appreciable frequencies, would be effective in significantly increasing the frequency of w^i reversions. We did not anticipate that an alkylating agent, such as ENU, would effectively delete the 2.9-kb duplication associated with the w^i mutation. The results obtained indicate that the w^i system, especially in the form $Dp(1:1:1:1)w^i$, detects a broad spectrum of mutagenic agents and can be profitably included in the test now used to determine whether or not chemical compounds are mutagenic. Judicious mutagenicity testing of a specific chemical compound requires that more than one test be employed before reaching a conclusion. The simplicity and low cost of raising *Drosophila* and the objectivity and sensitivity of the w^i system described here militate for inclusion of this system as a test of environmental mutagens.

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