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Cytogenetic and complementation analyses of recessive lethal mutations induced in the *X* chromosome of *Drosophila* by three alkylating agents

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SUMMARY

Salivary-gland chromosomes of 54 methyl methanesulphonate- and 50 triethylene melamine-induced *X*-chromosome recessive lethals in *Drosophila melanogaster* were analysed. Two of the lethals induced by the monofunctional agent and 11 of those induced by the polyfunctional agent were found to be associated with detectable aberrations. A complementation analysis was also done on 82 ethyl methanesulphonate- and 34 triethylene melamine-induced recessive lethals in the *zeste-white* region of the *X* chromosome. The EMS-induced lethals were found to represent lesions affecting only single cistrons. Each of the 14 cistrons in the region known to mutate to a lethal state was represented by mutant alleles, but in widely different frequencies. Seven of the TEM-induced lethals were associated with deletions, only one of which had both breakpoints within the mapped region. Twenty-six of the 27 mutations in which only single cistrons were affected were mapped to 7 of the 14 known loci. One TEM- and two EMS-induced mutations were alleles representing a previously undetected locus in the *zeste-white* region.
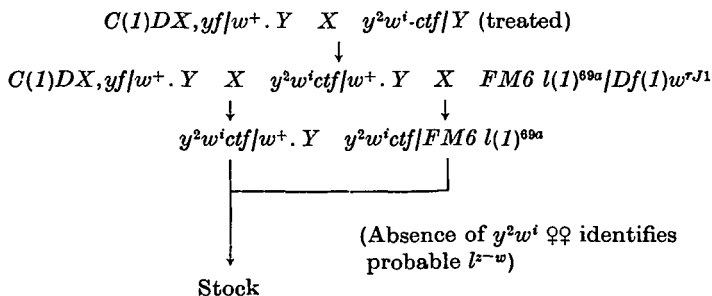
1. INTRODUCTION

The deletion of small segments of eukaryotic chromosomes has been found to occur spontaneously and also to be induced by a variety of physical and chemical agents. These deletions appear to constitute a class of mutational alteration distinct from either point mutations or gross aberrations related to chromosome breakage. The frequencies of small deletions and other small chromosomal alterations induced by ionizing radiations show a linear increase with dose (Muller, 1940). This is assumed to reflect breakage of the chromosome at two points lying in proximity to one another in a single ionization path. The limited data on frequencies of small deletions in relation to dose of effective chemical agents indicate that this relationship is also linear. The origin of such deletions induced by polyfunctional alkylating agents has been attributed to interference with replication of a segment of the DNA (Fahmy & Fahmy, 1955; Lim & Snyder, 1968). It is unlikely that these alterations are related to chromosome breakage, since they have not been found in association with recessive lethal mutations induced by ethylenimine, a monofunctional alkylating compound effective in breaking chromosomes (Lim & Snyder, 1968).

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Our earlier investigation of the mutagenic effects of ethylenimine (EI) and ethyl methanesulphonate (EMS) suggested that such monofunctional agents differ from polyfunctional agents (e.g. triethylene melamine [TEM] or nitrogen mustard) in being unable to induce small deletions in the chromosomes of *Drosophila melanogaster* (Lim & Snyder, 1968). Lifschytz & Falk (1969) subsequently reported that about 20 % of a group of recessive lethals induced with high doses of EMS in the *maroon-like* (*mal*) region of the *D. melanogaster* X chromosome were apparent deletions extending over two or more cistrons. We have re-examined the effectiveness of EMS, methyl methanesulphonate (MMS) and TEM in the induction of small deletions after treatment of mature spermatozoa of *D. melanogaster*. These experiments have involved cytogenetic examination of X-chromosome recessive lethals and complementation analyses of lethals induced in the *zeste-white* region of the chromosome. The results obtained indicate that deletions in euchromatic regions of the *Drosophila* genome are produced in very low frequencies by monofunctional alkylating agents.

2. MATERIALS AND METHODS

In experiments for the induction of X-chromosome recessive lethals for cytogenetic analysis, 4 mM and 7·5 mM MMS (Aldrich Chemical Co.) in 0·05 M Tris made 0·4 % with NaCl and 0·15 mM TEM (Imperial Chemical Industries Ltd.) in 0·4 % NaCl were used. One-day-old *D. melanogaster* males of the constitution $y^2w^1ct^6f/sc^8 . Y . B^S$ were injected with about 0·3 μl of the appropriate mutagenic solution. Injected males were allowed to recover for 12–24 h and were then mated for 3 days with $y\ sc^{S1}\ In49\ sc^8$; $dp\ bw$; $st\ p^P$ virgin females. The procedures used in maintaining the lethals obtained, determining their approximate genetic locations, and in preparing salivary-gland chromosomes for examination have been described (Lim & Snyder, 1968).

Recessive lethals in the *zeste-white* region were induced by feeding males collected less than 24 h after eclosion on a 25 mM EMS (Eastman Kodak Co.) solution in sterile 1 % sucrose by the method of Lewis & Bacher (1968) or by injecting 1-day-old males with about 0·3 μl of 0·1, 0·15 or 0·2 mM TEM in 0·4 % NaCl. The TEM used in these experiments was a gift from Lederle Laboratories Division of the American Cyanamid Co. The basic mating scheme used for the selection of lethals in the region was the following modification of the scheme used by Judd, Shen & Kaufman (1972):

$$C(1)DX,yf/w^+ . Y \quad \text{X} \quad y^2w^i\text{-}ctf/Y \text{ (treated)}$$
$$\downarrow$$
$$C(1)DX,yf/w^+ . Y \quad \text{X} \quad y^2w^ictf/w^+ . Y \quad \text{X} \quad FM6\ l(1)^{69a}/Df(1)w^{rJ1}$$
$$\downarrow \qquad\qquad\qquad\qquad \downarrow$$
$$y^2w^ictf/w^+ . Y \quad y^2w^ictf/FM6\ l(1)^{69a}$$

(Absence of y^2w^i ♀♀ identifies probable l^{z-w})

Stock

The females used in this scheme were obtained from crosses in which they were automatically virgins because their male sibs had lethal combinations of chromosomes: i.e.

$$C(1) \ DX,yf/Y \times Df(1)w^{rJ1}/w^+ . \ Y \quad and \quad FM6 \ l(1)^{69a}/l(1)^{t2+14a} \times Df(1)w^{rJ1}/w^+ . \ Y.$$

The $Df(1)w^{rJ1}$ deletion for the 3A2–3C2 region, obtained from Dr B. H. Judd, is balanced by the $w^+ . Y$ chromosome in which the 2D1-2 to 3D3-4 section of the X chromosome has been inserted into the long arm of the Y chromosome. The $l(1)^{t2+14a}$ recessive lethal, located at about 65·0, was obtained from Dr W. D. Kaplan, and $l(1)^{69a}$ is a lethal in the $FM6$ balancer X chromosome located outside the 2D1-2 to 3D3-4 region that we induced with EMS. The $C(1)DX,yf$ and $FM6$ chromosomes are described by Lindsley & Grell (1968).

After the lethals were established in stock cultures, each of them was again tested against the $Df(1)w^{rJ1}$ deletion to confirm that the induced mutation was in the *zeste-white* region. The mutants were then mated to stocks carrying recessive lethal alleles of each of the 14 loci identified by Judd *et al.* (1972) in that region. The mating scheme used in these crosses was the following, $FM6 \ l(1)^{79a}/l(1)^{z-w, \ Judd} \times l(1)^{induced}/w^+ . \ Y$, where complementation between the two mutants in the region is indicated by the presence of B^+/B^+ females and non-complementation by the absence of this class in the progeny. We are indebted to Dr Judd for the following recessive lethal mutant alleles of the 14 loci: *13z (gt), k11 (tko), b22 (zw1), g10 (zw8), d28 (zw4), h10 (zw10), c21 (zw2), b24 (zw3), e5 (zw6), k3 (zw12), e3 (zw7), j1 (zw5), a5 (zw11)* and *k18 (zw9)*. For the detection of possible alleles at the *zeste* locus, each of the induced lethals was also tested against $y^{59b}z$ obtained from Dr M. M. Green.

3. RESULTS

(i) *Cytogenetic analyses of* X-*linked lethals*

Recessive lethals induced by MMS and TEM (Table 1) were obtained to extend our earlier work on the frequencies and nature of chromosomal aberrations associated with chemically induced mutations. The methylating agent, MMS, was used because Fahmy & Fahmy (1961) had reported that about 17 % of the X-chromosome lethals induced by injection of males with a 4·5 mM concentration of this agent are associated with cytologically detectable small deletions. In the mutagenic treatment used by these workers, lethals were induced with a frequency of 8·5 %. Since our objective was quantitation and characterization of deletions induced by the agent, we injected males with 7·5 mM MMS to obtain a somewhat higher level of genetic damage, as measured by the incidence of sex-linked lethals (11·6 % in our experiments).

Genetic map locations were estimated for 54 of the MMS-induced lethals that were selected as complete lethals, and the salivary-gland chromosomes of these stocks were examined. One of the mutations, $M20$, was found to be associated with an inversion having breakpoints at 2B9-11 and 3C4-6. In addition, one small deletion was detected; associated with lethal $M54$ (estimated map location 0·56 $\pm$ 0·18) and extending from 2B10 through 2B18. The remaining 52 lethals were free of cytologically detectable aberrations.

Fifty of the TEM-induced lethals listed in Table 1 also were located genetically and examined cytologically. Of these, three were associated with sizeable inversions and an additional eight with small deletions. The mutant stocks in which such deletions were detected in the region of the lethal were as follows: *T3* (1A1-2; 1A7-8), *T4* (1A1-2; 1A5–6), *T9* (11D3-5; 11D10-E1 and 12A2-4; 12A9-B1), *T19* (1A1-2; 1A8-B1), *T24* (16A6-B1; 16B2-4), *T30* (3C6-7; 3C8-9), *T34* (2C2-4; 2C10-D1), and *T36* (11A3-5; 11A10-11).

Table 1. *Sex-linked recessive lethals and translocations involving the* Y, II *and* III *chromosomes in progenies from males injected with 0·3 μl of MMS or TEM in the indicated concentrations*

		X-chromosome lethals			Translocations		
Expt	Treatment	Tested	No.	Freq. (%)	Tested	No.	Freq. (%)
1	7·5 mM MMS	1167	134	11·6	1158	8	0·73
2	4·0 mM MMS	1148	32	2·8	1087	0	—
3	0·15 mM TEM	867	84	9·7	—	—	—

Table 2. *Chromosomal aberrations associated with MMS- and TEM-induced lethals*

		Inversions		Deletions	
Treatment	Tested	No.	Freq. (%)	No.	Freq. (%)
7·5 mM MMS	54	1	1·8	1	1·8
0·15 mM TEM	50	3	6·0	8	16·0

Note that two deletions were detected near the estimated genetic location (39·9 ± 0·5) of the *T9* lethal. Of the eight deletions found, three involve the loss of bands near the tip of the chromosome. The X chromosome in the stock we used has two darkly staining bands of medium size located between the cap (a grey band that we interpret as 1A1) and the prominent 1A5,6 doublet. We interpret these darkly staining bands to be 1A2 and 1A3.

The chromosomal aberrations found in association with the MMS- and TEM-induced lethals are summarized in Table 2. The incidence of deletions related to treatment with the monofunctional agent is much lower than the 17 % reported by Fahmy & Fahmy (1961) from experiments yielding a lower frequency of lethals. Small deletions induced by TEM also were found in a much lower frequency than the 43 % reported by Fahmy & Fahmy (1956) from experiments in which males were injected with the same concentration of the chemical. Our results with the polyfunctional agent are in much closer agreement with those of Slizynska & Slizynski (1947), who found that 19 % of X-chromosome lethals induced by chemicals and 20·8 % of those induced by X-radiation were associated with deletions.

(ii) *Analysis of lethals in the* zeste-white *region*

In our experiments, using the genetic screen to select for non-leaky recessive lethals, slightly less than 1 % (90/9794) of the tested X chromosomes that had been treated with 25 mM EMS carried a lethal in the *zeste-white* region (Table 3). This segment contains about 1 % of the DNA in the X chromosome (Rudkin, 1965) and represents about 1 % of the genetic map (0·75/70 map units). Eight of the 90 mutants were characterized by male sterility, presumably because of the involvement of loci outside the region of interest. All of the remaining lethals behaved as

Table 3. *Recessive lethal mutations in the* zeste-white *region in progenies from males fed on EMS or injected with 0·3 µl of TEM in the indicated concentrations*

| | | Lethals in the z-w region | | |
Expt	Treatment	Tested	No.	Freq. (%)
4	25 mM EMS	976	6	0·61
5	25 mM EMS	3419	34	0·99
6	25 mM EMS	5399	50	0·93
7	0·1 mM TEM	5148	7	0·14
8	0·1 mM TEM	4901	7	0·14
9	0·1 mM TEM	6174	16	0·28
10	0·15 mM TEM	608	1	0·17
11	0·2 mM TEM	865	3	0·35

single-site mutations. Their distribution among the complementation groups that have been established in the *zeste-white* region of the chromosome is shown in Fig. 1. Two of the EMS-induced lethals, *e50* and *e77*, are not included in Fig. 1. These mutations were found to be allelic, and also to be allelic to a third, TEM-induced, lethal. The three alleles show complementation with the test alleles at each of the *zw* loci, and represent a locus between *l(1)zw1* and *l(1)zw2* as determined by complementation behaviour with several deletions having breakpoints at known positions within the 3A2–3C2 region. The analysis of these mutations will be reported elsewhere.

One of the mutant stocks from EMS treatment, *e34*, had recessive lethals induced in two non-adjacent cistrons (*l(1)zw1* and *l(1)zw12*) within the *zeste-white* region. Two of the lethals were also found to be associated with visible mutations, *e83* with a mutation at the *vermilion* locus and *e95* with a *Notch* mutation.

In the experiments in which the mutagenic treatment consisted of the injection of TEM, lethals in the *zeste-white* region were detected in about 0·2 % (34/17,696) of the chromosomes tested. Seven of the 34 lethals obtained were found to be associated with deletions. The extent of these deletions within the chromosome region of interest, as determined by complementation behaviour in crosses with mutant alleles of each of the loci, is shown in Fig. 1. Only one of the deletions, identified as lethal *7*, has both breakpoints within the region. The deletion designated as lethal *1* complements with the *k18* allele of *l(1)zw9*, but is associated with a zeste mottling of the eyes in *l/k18* (*z sp1 sn³*) heterozygous females. The distri-

bution of 26 of the 27 TEM-induced lethals found to involve single cistrons is shown also in Fig. 1. One of these lethals, *214*, affecting only the *zw1* locus, is inseparably associated with a translocation having cytologically determined breakpoints at 3A4-6 in the *X* chromosome and 94F in the right arm of the third chromosome. The other single-locus mutants were found to be free of detectable chromosomal aberrations. The lethal (*13*) not included in Fig. 1 is the one referred to above that is allelic with the *e50* and *e77* EMS-induced lethals and complements with alleles of each of the *zw* loci.

Selection of the mutants in these experiments was for non-leaky recessive lethals,

gt	tko	z	zw1	zw8	zw4	zw10	zw2	zw3	zw6	zw12	zw7	zw5	zw11	zw9	w

EMS-induced

gt	tko	zw1	zw8	zw4	zw10	zw2	zw3	zw6	zw12	zw7	zw5	zw11	zw9
e79	e75	e2	e5	e11	e36	e1	c6	e12	e34	e13	e32	e22	e51
		e4	e17	c48	e87	e3	e28	e72		e30	e55		
		e9	e35	e57	e90	e7	e29	e80		e42	e93		
		e10	e45	e82		c8	e56			e92			
		e15	e65	e85		e14	e60						
		e16	e78			e20							
		e18	e88			e27							
		e24				e31							
		e25				e37							
		e33	e34			e41	e43						
		e40	e49			e44	e46						
		e52	e54			e47	e53						
		e67	e70			e59	e61						
	e76	e83	e84		e66	e68	e71						
	e89	e94	e95		e73	e74	e81						

TEM-induced

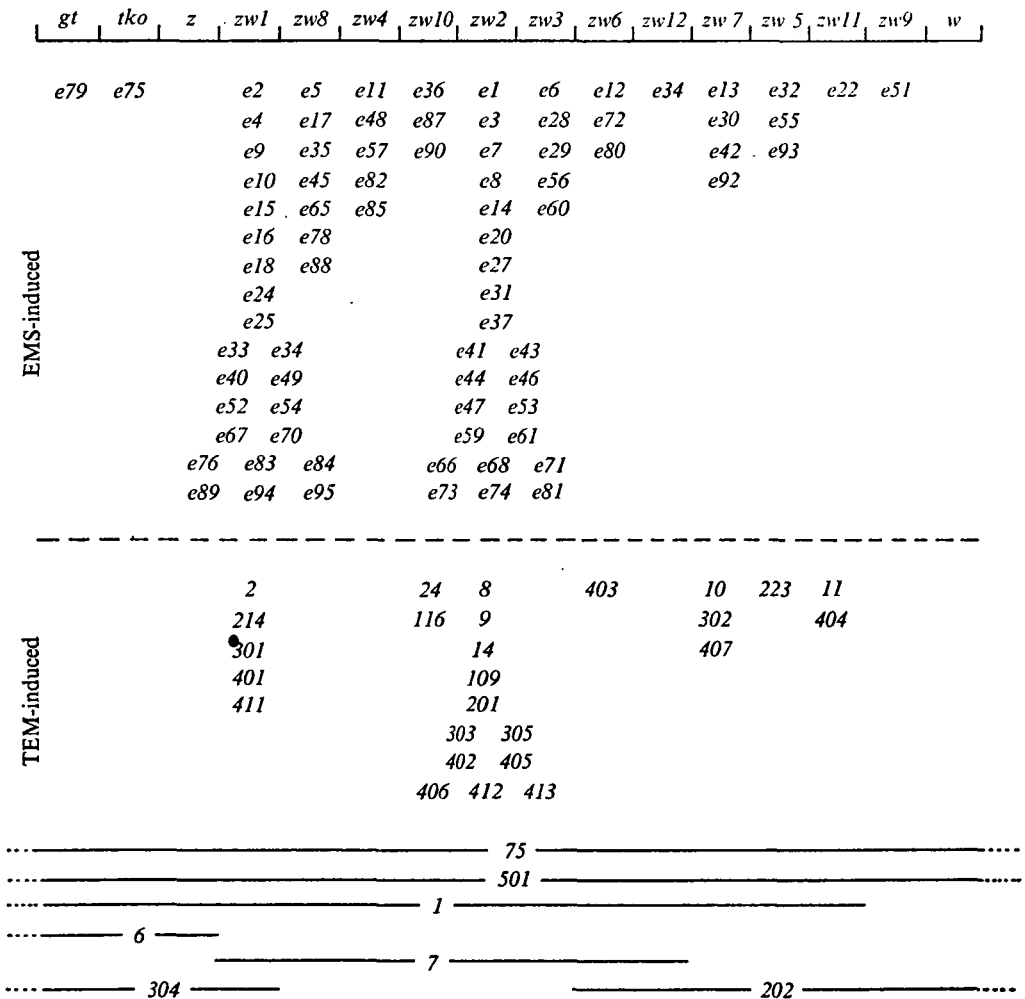| zw1	zw10	zw2	zw6	zw7	zw5	zw11
2	24	8	403	10	223	11
214	116	9		302		404
•301		14		407		
401		109				
411		201				
		303 305				
		402 405				
	406 412 413					

Extent of TEM-induced deletions:
75
501
1
6
7
304
202

Fig. 1. Complementation map of lethals in the *zeste-white* region induced in mature spermatozoa by ethyl methanesulphonate and triethylene melamine. The region, from 3A1 (*gt*) to 3C2 (*w*), is defined by the *Df(1)w^{rJ1}* deletion. All of the EMS-induced mutations affect single cistrons, although one of them, *e34*, had lethal lesions in both the *zw1* and *zw12* cistrons. The horizontal lines indicate the extent of the TEM-induced deletions detected.

although three of the EMS-induced mutations (*e52, e78* and *e82*) subsequently were found to be semi-lethal, i.e. to result in a low frequency of females homozygous for the given mutation. Because of the selection applied, it is not readily possible to draw any conclusions concerning the relative mutabilities of the several loci. Four of the loci are represented by single lethal alleles. Judd *et al.* (1972) reported only two semilethal mutant alleles at one of these loci, *l(1)zw9*, and a low frequency of mutants at the other three. Similarly, we found, as did Judd *et al.*, that relatively large numbers of the mutants obtained were alleles at *l(1)zw1* or *l(1)zw2*.

Judd *et al.* (1972) concluded that the *gt* locus is located in 3A1, a chrommere that they found was not deleted in *Df(1)w^{rJ1}*. The two mutant alleles of *gt* that were included in their investigation were found to be non-complementing with three deletions in which the chromomere is reduced or missing. An inconsistency that they were unable to explain was the reduced viability of one of the mutant alleles (*E6*) and greatly reduced viability of the second allele (*13z*) in heterozygotes with *Df(1)w^{rJ1}*. In our experiments, the only lethal mutant allele of *gt* was detected using this deletion as the genetic screen. Heterozygotes of this allele (*e79*) with either *Df(1)w^{rJ1}* or the *13z* allele of Judd *et al.* are inviable. These data indicate that part of the *gt* cistron is included in the *Df(1)w^{rJ1}* deletion.

Five mutant alleles of the *l(1)zw10* locus were obtained by Judd *et al.* (1972) and found to be semilethal in males and homozygous females. Each of the alleles also resulted in sterility in both sexes. From the map position and mutant phenotype, it was suggested that the locus may be synonymous with *abe*, abnormal eye, described by Fahmy & Fahmy (1959). We have obtained five lethal mutant alleles of *l(1)zw10*: *e36, e87* and *e90* induced with EMS and *24* and *116* induced with TEM. When each of these alleles was used in crosses with the *h10* allele of Judd *et al.*, the resulting females included a low frequency (2–25 %) of those heterozygous for the two alleles. Expression of the mutant phenotype is quite variable in these stocks, but many of the surviving heterozygous mutant females from each of the crosses have eyes of reduced size and rough texture and/or wings that are atypical primarily in having the inner margins broken by large incisions. These females were also found to be sterile.

4. DISCUSSION

These results are consistent with our earlier hypothesis (Lim & Snyder, 1968) that the infrequency of small deletions induced by monofunctional alkylating chemicals represents a qualitative difference between the action of these agents and those with two or more reactive groups per molecule. It is not clear that there is a sensible distinction to be made, relative to the mechanisms involved in their production, between deletions involving the loss of one or a few bands and those extending over a greater length of a salivary-gland chromosome. Rudkin (1965) has estimated that there are about 5000 nucleotide pairs in the smallest measurable band and up to 10 times that amount of DNA in the largest bands. Relatively little is known about the organization of the DNA molecules in eukaryotic chromosomes; certainly too little to support any conclusions concerning the sizes of deletions that

could result from cross-linking by an alkylating moiety with two or more reactive groups or from two breaks in a chromosome traversed by a single ionization track. Indeed, an insight into the structural organization of DNA in a chromosome could be obtained from an investigation of the sizes of deletions induced by an agent such as TEM or nitrogen mustard under conditions where these aberrations have a high probability of resulting from alkylation by single molecules. A five-membered alkyl chain, such as that represented by nitrogen mustard, can span a distance of somewhat less than 8 Å (Lawley, 1961). Based on the sizes of the TEM-induced deletions recovered in the *zeste-white* region (Fig. 1), only one of which had both breakpoints within the region, this chromosome segment would not be long enough for use in such an investigation. The TEM-induced deletions detected in cytogenetic examination of the entire chromosome are considerably shorter than those detected in the *zeste-white* region, a phenomenon for which we have no explanation.

Various monofunctional alkylating compounds, usually in relatively high doses, have been found to break chromosomes in several eukaryotic systems (for review see Auerbach & Kilbey, 1971). The mechanisms involved in such breakage are not well understood. However, deletions of more than a few nucleotide pairs resulting from alkylation by monofunctional agents are expected to represent '2-hit' aberrations. In view of the expected distribution of such breaks, the EMS-induced deletions reported by Lifschytz & Falk (1969) show a very unusual distribution of sizes. More than half of these deletions were mapped to two or three cistrons each. Six of these mutants and five others induced by X-radiation and identified as two-cistron deletions in complementation mapping of the *mal* region have been re-examined and found to represent lesions in only single cistrons (Schalet, 1972).

Six of the 70 EMS-induced lethals mapped in the *mal* region by Lifschytz & Falk (1969) were associated with deletions large enough to be detected easily in cytological examination of the polytene chromosomes; i.e. it is unlikely that our finding of very few deletions in association with EI-, EMS- and MMS-induced lethals is related to resolving power of the cytological technique used. Moreover, no deletions have been detected in complementation analysis of 82 EMS-induced lethals in the *zeste-white* region (Fig. 1), and B. H. Judd (personal communication) has found none associated with some 40 additional EMS-induced lethals in the same region. Similarly, Hochman (1971) found only a single mutation that appeared from genetic tests to be a deletion in complementation analysis of 70 EMS-induced lethals in chromosome 4. The *mal* region investigated by Lifschytz & Falk (1969) is adjacent to the basal heterochromatin of the X chromosome, and three of the six large deletions they detected had proximal breakpoints in the heterochromatin. Other observations relating heterochromatin to chemically induced chromosome breakage and deletions are those of Bishop & Lee (1969, 1973), on EMS-mutability of y^+ and ac^+ located normally in X-chromosome euchromatin and when translocated to the Y chromosome, and Williamson (1970) on EMS-mutability of male fertility loci in the Y chromosome. The previously cited results obtained by Hochman (1971) from complementation analysis of induced lethals in chromosome 4, on the other hand,

give no indication of such an effect of heterochromatin on chemically induced deletions.

The data from our experiments lend substantial support to the conclusion by Judd *et al.* (1972) that one functional unit in the chromosome region examined can be related to each chromomere in the polytene chromosome. Thus, 81 EMS-induced mutations (including the two in non-adjacent cistrons in the *e34* stock) and 26 TEM-induced mutations affecting only single cistrons, can be assigned without ambiguity to specific functional units described by these investigators. There is some uncertainty about the precise number of bands in this region of the polytene chromosomes. Our finding of three mutant alleles representing another cistron in the region capable of mutating to a state lethal to the developing organism is not inconsistent with, and probably strengthens, the thesis of 1 function:1 chromomere. Judd *et al.*, for example, detect eight bands in the 3A subdivision of the chromosome, while Beerman (1972) gives a map, based on that of Bridges (1938) but modified according to subsequent results from electron microscopy, in which there are 9 bands in 3A. Preliminary data indicate that the additional cistron we have detected is located in that subdivision of the chromosome.

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