ON THE INTERPRETATION OF MUTAGENICALLY INDUCED MOSAICISM IN DROSOPHILA

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ABSTRACT

This paper draws attention to the formal parallelism that exists between chromosomal-loss mosaicism and mutagenically induced mosaicism in Drosophila and suggests that, although the underlying processes by which these two types of genetic mosaics are generated are very different, the more refined methodology employed in developmental analyses of genetically induced chromosomal-loss mosaics may be profitably extrapolated to mutagenesis studies. Results obtained from various studies of genetically induced mosaics and from a previous EMS mutation induction experiment at the yellow locus are utilized to illustrate this methodology and to estimate the total mutagenicity rates of EMS.—The following are some of the tentative conclusions that have been drawn in this report regarding an EMS concentration that produced 31% F_2 lethals in the standard *X*-linked recessive lethal test: (1) The frequency of cuticular mosaics is at least 5 times that of F_3 lethals. (2) At least 60% of all cuticular mosaics go undetected in the standard *X*-linked recessive lethal test since their mutant tissue does not extend into the germ line. (3) The frequency of EMS-induced cryptic mosaics is probably less than 10% the frequency of cuticular mosaics. (4) Some EMS-induced mutations are probably *bona fide* completes; if confirmed, this inference must be taken into consideration in estimating the total mutagenicity rates of this agent and in molecular interpretations of its mechanism of action. (5) The fact that the proportion of mutant tissue in EMS-induced mosaics is greater than 25% is consistent with the suggestion that the action of EMS is occasionally delayed until after the first cleavage division of the embryo. (6) Such an EMS concentration causes on the average more than 5 independent genetic alterations in the entire haploid genome of an *X*-bearing sperm.—This report clarifies the experimental evidence that must be generated, and the methodology that can be used to analyze this evidence, if it is of interest to render these and related conclusions regarding the effect of EMS on *D. melanogaster* more accurate, or if it is of interest to conduct a similar analysis for other mutagens that cause a significant degree of mosaicism.

SINCE the classical experiments of STURTEVANT (1929, 1932), and especially during the last decade, genetic mosaics have frequently been employed in developmental studies of Drosophila. Consequently, certain modes of analysis have been developed and applied to investigations of various developmental aspects of this organism (reviewed by GEHRING 1976). The purpose of this

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communication is to apply the methodology and some of the conclusions that have so far emerged from these developmental studies to another class of genetic mosaics: mosaics that exist among the descendants of flies treated with chemical and other mutagenic agents (*e.g.*, AUERBACH 1946; MATSUDAIRA *et al.* 1967). In particular, the ensuing analysis corroborates the already established (LEE, KIRBY and DEBNEY 1967; LEE, SEGA and BISHOP 1970) formal parallelism that exists between genetically and mutagenically induced genetic mosaics and suggests that some interpretations of data derived from mutation induction experiments with *D. melanogaster* need to be extended or revised.

Parallelism between chromosomal loss and mutagenically induced mosaicism

This section discusses some of the evidence for the existence of such a formal parallelism, along with some of its most immediate implications.

a. Cytological observations (PARKS 1936) suggest that the orientation of the axis of the spindle fibers of the first zygotic division with respect to the axes of the egg is indeterminate. As a result, the orientation of the mosaic boundary that separates mutant and nonmutant tissues in genetic mosaics is also indeterminate: left-right, fore-and-aft, and a variety of other arrangements are observed. As expected, this lack of preferential orientation of the mosaic boundary is observed in genetic mosaics induced by chromosomal loss (*e.g.*, GARCIA-BELLIDO and MERRIAM 1969; LEE, KIRBY and DEBNEY 1967) or by the chemical mutagen ethyl methanesulfonate (EMS; LEE, SEGA and BISHOP 1970; LEE 1976).

b. The frequently observed pattern of contiguous mutant and nonmutant cuticular patches in both types of mosaics (GARCIA-BELLIDO and MERRIAM 1969; LEE, SEGA and BISHOP 1970) is probably attributable to the fact that very little mixing of cleavage nuclei occurs during early embryogenesis. Hence, it appears that, in both chromosomal loss and mutational mosaics, the closer the two cells are on the blastoderm surface, the more likely they are to have an identical genotype (STURTEVANT 1929; GARCIA-BELLIDO and MERRIAM 1969).

c. A study of EMS-induced yellow (=*y*:*1*–0.0, body color yellow, hairs and bristles brown with yellow tips; can be distinguished from wild type in almost all cuticular regions) mosaics and their offspring indicated that the genotype of the germ nuclei was independent of the genotype of the sample of nuclei that gave rise to the head and that the composition of the germ line was not entirely independent of the composition of nuclei that developed into the abdominal cuticular segments (LEE, SEGA and BISHOP 1970). This conclusion has been reached by a series of χ^2 tests for independence. A more accurate approach is afforded now by calculating the distances in sturts between the germ cells and various cuticular landmarks (HOTTA and BENZER 1973). This procedure is preferable since it allows a more quantitative analysis of cell lineage relationships between the germ cells and various somatic structures. Such an analysis has been recently carried out (NISSANI 1977) with 320 fertile *Y*-chromosome mosaic males and females. This analysis disclosed that the nearest cuticular structures, in terms of cell lineage relationships, to the germ cells are the external genitalia and that, in general, the more posteriorly located a prospective cuticular struc-

ture is on the blastoderm surface, the more closely related it is to the germ cells. The close developmental relationship of the germ cells to the external genitalia, and consequently also to the Malphigian tubes (NISSANI 1975), may have some applications in developing an efficient screening system for induced transmissible visible mutations and in reversion studies.

d. It is well documented (*e.g.* LEE, KIRBY and DEBNEY 1967) that there is very little correlation between the proportion of mutant tissue in the germ line and the proportion of mutant tissue in the rest of the embryo. This observation can be readily explained as follows. The proportion of mutant tissue in the entire cuticle provides a good approximation of the proportion of mutant tissue in the adult (GARCIA-BELLIDO and MERRIAM 1969), and, therefore, it reflects the time during cleavage at which a mutation became fixed or at which chromosome loss occurred. In contrast, the relative size of a mutant patch in a mosaic germ line, or in any other larval or adult organ, is primarily determined by (1) the number of primordial cells which give rise to this organ, (2) the area that the progenitor cells of this organ occupy on the blastoderm surface, and (3) the manner in which this area is "intersected" by the mosaic boundary (for a review, see NISSANI and LIPOW 1977). Hence, the observed lack of correlation between the proportion of mutant tissue within any developmentally narrowly localized, germinal or somatic, mosaic organ and the proportion of mutant tissue in the entire cuticle is exactly what is expected on theoretical grounds.

Quantitative estimates of total mutagenicity rates

Estimates of mutation rates are imprecise when the effects of agents used for mutation induction are expressed mosaically. Hence, a scheme is needed that would make possible mutagenicity measurements of a given agent on individuals as well as on future generations (CARLSON 1964). Aside from estimating the actual effects of mutagenic agents, such a scheme is important for several reasons. First, doses of different agents are considered biologically equivalent if they bring about identical total mutation rates. Thus, in order to determine biological equivalence, the proportion of instances in which an agent caused gene mutations that do not extend into germinal tissues must be assessed. Second, to the extent that information gained from Drosophila is applicable to humans, mutations arising in nongerminal cells and which are not transmitted to future generations are nonetheless of significance in genetic counseling (HARTL 1971). Third, such estimates are of interest because there is a large body of experimental evidence that suggests that most potent mutagens are also carcinogens (AUERBACH 1976).

The most objective and convenient, and consequently the most widely employed, test for various mutagens in Drosophila is the *X*-linked recessive lethal test, which assays only germinal tissues. The total mutagenicity rates of a given agent must therefore be measured from a scheme that relates studies with specific visible mutations to those with the *X*-linked lethal test (CARLSON 1964). The underlying basic assumptions for such a scheme have already been discussed (CARLSON 1964). The following discussion will show that when a visible mutation such as yellow is employed, and when some recent advances in devel-

opmental studies of genetic mosaics are considered, Carlson's method of extrapolating information obtained from mutagenicity studies of mosaically expressed visibles to the *X*-linked lethal test can be extended and rendered more accurate.

1. *Analysis of flies with mosaic cuticle*: In a recent study (NISSANI 1977) mutant and nonmutant cuticular and germ line distributions of *pal*-induced (BAKER 1975) *Y*-chromosome *D. melanogaster* mosaic males were analysed. Counting mosaic discs as ½ mutant, the average proportion (p) of mutant cuticular tissue in a sample of 215 fertile mosaic males was 42.5%. This suggests that *pal*-induced chromosomal loss in these males most often occurred at the first cleavage division, but that occasionally the loss occurred later. Progeny tests of these 215 males revealed that 39.5% of the males had a completely mutant germ line, 16.7% of the males had a mosaic germ line and 43.7% had a completely nonmutant germ line. These results suggest that mosaicism induced by an agent which causes a similar average proportion of mutant tissue should also have a similar germ line distribution. In order to apply these results to the *X*-linked recessive lethal test of a given mutagen, it is necessary to obtain an estimate of p for that particular agent. Such information is available for EMS (LEE, SEGA and BISHOP 1970), and the analysis in this and the following sections will be largely limited to analysing the effects of this particular chemical mutagen. It should, however, be emphasized that the main purpose of the following exposition is to refine the methodology employed in analyses of the effects of all mutagens that cause a significant degree of mosaicism. EMS is used here merely for illustrative purposes and because a sufficient amount of experimental evidence is available. Should it become of interest to determine the total mutagenicity rates (and related aspects) of other mutagens, or to obtain more precise estimates of EMS action than those presented here, then the following discussion points to the experimental evidence that must be generated and delineates the methodology that may be employed in interpreting these data.

LEE, SEGA and BISHOP (1970) reported that p for an EMS concentration that produced 31% F_2 lethals was approximately 35%. This p value seems sufficiently similar to the $p = 42.5\%$ reported for *pal*-induced mosaics (NISSANI 1977) that extrapolating from the germ line distribution among *pal*-induced mosaics may provide reasonably close approximations of the corresponding distribution of EMS-induced *X*-linked lethals. Assuming that different concentrations of EMS produce similar p values, the fact that less than ⅕ of *pal*-induced mosaic males had a mosaic germ line suggests that for a given EMS dose that causes a certain frequency of F_3 lethals (based on progeny tests of a large sample of F_2 females per F_2 culture), the frequency of cuticular mosaics is at least 5 times higher. This is a minimal estimate for several reasons. First, p among *pal*-induced *Y*-chromosome mosaics was higher than p for EMS-induced y mosaic mutations and hence more EMS-induced mosaics will go undetected in the lethal test, since when p is lower the mutant patch in a given mosaic is less likely to extend into the germ line. Second, double mosaics are often misclassified as completely mutant (EPLER 1966), and double or multiple hits of the same strand are usually recorded only as single hits. Similarly, after correcting data on F_2 lethals for

the occurrence of double mosaics, double or multiple hits of the same strand and *bona fide* completes (see below), it may be also estimated from the data of *pal*-induced mosaicism that for such a corrected frequency of EMS-induced completes, the frequency of all flies that are cuticular mosaics is at least double that frequency.

The data on *pal*-induced cuticular mosaics disclose also that the average proportions of mutant, nonmutant or mosaic germ cells are very similar to the respective average proportions in the entire cuticle. Indeed, theoretical considerations (*e.g.* NISSANI and LIPOW 1976) suggest that the only expected differences would be the result of sampling errors and the relatively high frequency of germinal mosaicism (as compared to frequencies of intradisc mosaicism of most other imaginal discs). The fact that p for the entire cuticle provides a close approximation of the proportion of mutant germ line in mosaic flies is very useful in interpreting experimental results obtained with the recessive lethal test. For instance, if it is found that a given mutagen causes a mutation to be fixed on the average at the third cleavage division (*i.e.*, p=12.5%), there is roughly a 12.5% probability that the mutant patch will extend into any narrowly localized area on the blastoderm surface, including the prospective germ cells region. Such a mutagen will therefore go undetected in the *X*-linked lethal test in approximately 88% of the treated chromosomes in whose replicas this agent caused a lethal mutation.

These lines of reasoning can provide more accurate estimates when they are applied directly to information obtained from 55 EMS-induced fertile yellow mosaic females (LEE, SEGA and BISHOP 1970). In this sample, p was 35%; 30.9% of mosaic flies had mutant germ line (due to the small number of offspring of some mosaic females in this category, it is assumed as a crude approximation here that one female in this category was in fact germinally mosaic), 9.1% had mosaic germ line and 60% had nonmutant germ line. Thus, it may be estimated from this limited sample that the frequency of EMS-induced cuticular mosaics is roughly 11 (1/0.091) times greater than the frequency of F_3 lethals (based on a large sample of females per F_2 culture), and 3 (1/0.309) times that of complete mutants (after a correction for two or more hits on the same or on two complementary strands and for *bona fide* completes is made). In addition, these data again corroborate the assertion that, if an estimate of cuticular mosaics with completely nonmutant germ line were made directly from the average proportion of nonmutant cuticular tissues in this sample, this estimate would have provided a close approximation of the actual value: the average proportion of nonmutant imaginal discs (excluding mutant and mosaic) among these 55 fertile cuticular mosaics was 63.7% (calculated from Table II, LEE, SEGA and BISHOP 1970). Therefore, one could predict that when germinal tissue is assayed in the *X*-linked lethal test in both the F_2 and F_3 generations, the frequency of undetected cuticular mosaics is also 63.7%. Indeed, the more direct estimate, which is derived from the frequency of nonmutant germ lines among 55 cuticular *y* mosaics, is very close: 60%.

An alternative approach for relating results obtained from mutation induction

of visibles to the lethal test has been proposed (LEE, KIRBY and DEBNEY 1967; LEE, SEGA and BISHOP 1970; LEE 1976). This approach led to quantitative estimates of EMS action which are in disagreement with the estimates presented at the preceding paragraph. Thus, this approach led to the conclusions that: (1) 76% of all mosaic embryos contained completely nonmutant germ lines and therefore would be missed by the lethal test, (2) p of EMS-induced mosaics is 20%, and (3) the frequencies of mutant and mosaic germ lines among these mosaics are roughly equal. In the present communication, the respective values drawn from the analysis of EMS-induced γ mosaics were 60% and 35%, and the analysis suggested that, for an EMS dose that produces 31% F_2 lethals, the frequency of germinal complete mutations is considerably higher than the frequency of germinal mosaics. But the methodology employed in computing the former estimates was based in part on the heuristic assumption that considering the germ nuclei as if they are selected at random from the total pool of cleavage nuclei can lead to meaningful quantitative estimates. Nevertheless, since the germ nuclei, as well as other groups of nuclei that give rise to specific narrowly localized regions, are developmentally very closely related, this approach is probably not very useful and should not be further employed in mutagenesis studies. The fact that p is greater than 25% also argues against the possibility that each locus consists of two double-stranded DNA molecules (LEE, SEGA and BISHOP 1970). Instead, the delayed action hypothesis of EMS (LEE, SEGA and BISHOP 1970), which is essentially analogous to the one used to explain mosaicism induced by chromosome loss (*e.g.*, GARCIA-BELLIDO and MERRIAM 1969; GELBART 1974), appears to afford the more likely explanation.

2. *Analysis of cryptic mosaics*: In making estimates of total mutagenicity rates one must also take into account, in addition to the class of cuticular mosaics dealt with above, the possibility that some flies whose entire cuticle is scored as completely mutant or completely nonmutant may nonetheless have some internal tissues of unlike genetic constitution. When p is roughly equal to 50%, several independently derived estimates indicate that the frequency of such cryptic mosaics is 6–10% (HOTTA and BENZER 1973; KANKEL and HALL 1976; NISSANI and LIU 1977). Although p for EMS is only 35%, 10% or less probably still provides a close enough approximation of the total frequency of cryptic mosaics induced by EMS. In addition, some cryptic mosaics (germinal mutant and cuticular nonmutant) are expected to be detected in the conventional lethal test. Thus, it may be tentatively concluded that the actual frequency of EMS-induced cryptic mosaicism is probably less than 1/10 the frequency of cuticular mosaicism.

3. *Can EMS induce bona fide complete mutations*? The preceding analysis pointed to the conclusion that most EMS-induced mutations that are scored as germinally complete in the X-linked recessive lethal test are in fact mosaically expressed mutations. As argued above, the reasons for believing this are the frequent occurrence of double mosaics (EPLER 1966) and the fact that most EMS-induced genetic mosaics have nonmosaic germ lines. Nevertheless, it is important to know whether or not EMS, as well as other mutagens, is capable of induction

of true complete mutations. An estimate of the frequency of such mutations is needed for quantitative estimates of total mutagenicity rates and, in addition, it has important implications for understanding the molecular mechanisms of action of various mutagens (AUERBACH 1976; NOVITSKY 1976). LEE, SEGA and BISHOP (1970) reported that the entire cuticle of 14% of all EMS-induced *y* mutants was completely *y*. The arguments presented in the preceding section argue against (but do not rule out) the possibility that this 14% frequency is largely attributable to cryptic mosaicism, *i.e.*, to the fact that some of the internal tissues of these apparent *y* completes had a y^+ genotype. Nor does it appear very likely that this 14% frequency can be explained by two independent hits in the two complementary strands of the DNA molecule at the *y* locus because, as will be shown later, the probability of a single hit in this locus with an EMS dose that produced 31% F_2 lethals is approximately 0.002. (However, this last conclusion remains to be experimentally verified, since it is *a priori* possible that, when a chemical mutagen acts upon one DNA strand, it is much more likely to have acted upon the complementary strand in the same region). Despite these uncertainties, it may be tentatively concluded that some EMS-induced F_2 lethals are *bona fide* completes (see also JENKINS 1967) and must be taken into consideration in quantitative assessments of the lethal test. Likewise, the tentative conclusion that some EMS-induced "complete" mutations are not due to a wholly mutant germ line in an otherwise mosaic individual or to double mosaicism, but that they are *bona fide* completes must be also taken into consideration in molecular hypotheses of EMS action in eukaryotes (AUERBACH 1976; DRAKE and BALTZ 1976).

4. *Number of hits per haploid* X*-bearing sperm*: In addition to the effect that given doses of particular agents exert on a single locus or on a single chromosome, it is of interest to determine their effect on the entire genome of mutagenized germ cells. It has been shown above that the total effect on the *X* chromosomes of treated flies of an EMS dose that generates a given frequency of F_3 lethals is at least 5 times that frequency. Assuming that the *X* constitutes about 1/5 of the entire haploid genome (BRIDGES 1938) and that all 4 chromosomes are equally susceptible to the action of EMS, the total mutagenicity rate for the whole genome increases to about 25 (5×5) times the frequency of F_3 sex-linked lethals. Thus. an EMS concentration that generates a frequency of 10% F_3 lethals is expected to produce on the average 2.5 or more hits per EMS-treated *X*-bearing sperm. It is not yet possible to derive by this method accurate estimates of the effects of specific EMS doses on the entire genome. Nevertheless, when the data of EPLER (1966) on the incidence of autosomal double mosaics are considered along with the expected frequencies of cryptic mosaics, *bona fide* completes and two or more hits on the same DNA strand, it appears likely that for an EMS concentration that produced 31% F_3 lethals (LEE, SEGA and BISHOP 1970), the actual number of hits per *X*-bearing sperm is greater than 5.

Another estimate is available from recorded frequencies of mutation induction at specific visible loci. Thus, an EMS concentration that induced 31% F_2 *X*-linked recessive lethals induced also 114 *y* mutations among a total of 50,243

chromosomes. At this EMS dose then, the frequency of mutation induction at the y locus was approximately 0.002 (LEE, SEGA and BISHOP 1970). The corresponding figure for the X-linked white locus, which was monitored in the same experiment, was more than double (when cryptic mosaicism is entered into the calculation of these frequencies). Taking the lower mutability estimate, and assuming that the y locus is not more mutable on the average than other loci and that there are approximately 5000 loci in the Drosophila genome (ALIKHANIAN 1937; BRIDGES 1938), the number of hits per X-bearing sperm with an EMS dose that produced 31% F_2 X-linked lethals may be estimated as 10 (0.002×5000). The discrepancy between this estimate (10) and the previous one (>5), as well as the limited amount of information upon which these estimates are based, suggests that at present such estimates can only provide order-of-magnitude approximations of the actual value.

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