Gonadal Mosaicism Induced by Chemical Treatment of Sperm in *Drosophila melanogaster*

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ABSTRACT Accurate interpretation of forward genetic screens of chromosomes exposed in mature spermatozoa to a mutagenic chemical requires understanding—incomplete to date—of how exposed chromosomes and their replicas proceed through early development stages from the fertilized ovum to establishment of the germline of the treated male's offspring. We describe a model for early embryonic development and establishment of the germline of *Drosophila melanogaster* and a model-validating experiment. Our model proposes that, barring repair, DNA strands modified by treatment with alkylating agents are stable and mutagenic. Each replication of an alkylated strand can result in misreplication and a mutant-bearing daughter nucleus. Daughter nuclei thenceforth replicate faithfully and their descendants comprise the embryonic syncytium. Of the 256 nuclei present after the eighth division, several migrate into the polar plasm at the posterior end of the embryo to found the germline. Based upon distribution of descendants of the alkylated strands, the misreplication rate, and the number of nuclei selected as germline progenitors, the frequency of gonadal mosaicism is predictable. Experimentally, we tracked chromosomes 2 and 3 from EMS-treated sperm through a number of generations, to characterize autosomal recessive lethal mutations and infer gonadal genetic content of the sons of treated males. Over 50% of 106 sons bore germlines that were singly, doubly, or triply mosaic for chromosome 2 or chromosome 3. These findings were consistent with our model, assuming a rate of misreplication between 0.65 and 0.80 at each replication of an alkylated strand. Crossing treated males to mismatch-repair-deficient females had no apparent effect on mutation rate.

KEYWORDS male sterility; meiotic mutants; paternal effect mutants; spermatogenesis

SINCE “The artificial transmutation of the gene” (Muller 1926), it has been noted that newly induced mutations occasionally appear as mosaics in which only part of the mutant animal expresses the new mutation. The mutant tissue sometimes extends into the germline. Muller attributed this to a duality of the genetic material in the sperm, when only one of the two components is mutated. During the Second World War, Charlotte Auerbach (1946a,b) performed classified experiments using *Drosophila melanogaster* looking for genetic effects of mustard gas. Hers was the first demonstration of chemical mutagenesis. She also found mosaics, and with significantly higher frequencies than found following X irradiation. The nature of the duality of the genetic contents

of mature spermatozoa remained enigmatic until the discovery of the double helix (Watson and Crick 1953a,b). Since that time many studies have attempted to explain results of chemical mutagenesis in terms of alterations of the DNA at the level of its individual strands, but have not fully characterized the effects on the germline. Such characterization is critical to the accurate interpretation of experimental data from forward genetic screens.

Mosaicism appeared to be a likely explanation for unexpected results obtained by Charles Zuker and his colleagues when they attempted to produce a collection of *D. melanogaster* stocks carrying, in balanced condition, homozygous-viable autosomes derived from males that had been exposed to very high levels of ethyl methanesulfonate (EMS) (Koundakjian *et al.* 2004). These lines were developed for use in screening for new mutations in nonvital genes.

Two series were produced: 6178 lines with mutagenized second and 6237 with mutagenized third chromosomes. The second chromosome lines, for example, were produced by exposing *cn bw* males to a heavy dose of EMS and crossing

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them to *CyO/Sco* females. *CyO/cn bw* sons were backcrossed individually to generate *CyO/cn bw* F₂ sons and daughters. The F₃ progeny resulting from inbreeding the *Cy/cn bw* F₂ were examined for their ability to produce *cn bw* homozygotes. At the dose of EMS employed, ~84% of the F₃ progenies contained no *cn bw* homozygotes; i.e., they were heterozygous for a recessive lethal mutation and were discarded. The lines with surviving *cn bw* flies were assumed to be homozygous for the mutagenized second chromosome and were retained as balanced stocks. An independent chromosome 3 screen was carried out using mutagenized *bw*; *st* males and *TM6b*, *Tb*-bearing females. Results were similar to those observed for chromosome 2.

Over time, however, stocks presumed to be homozygous viable began testing as homozygous lethal. Koundakjian *et al.* (2004) attributed this to the fixation of lethal mutations in lines descended from mosaic sons of the treated male; although the *cn bw* sons were homozygous for *cn bw*, some lines contained two different *cn bw* chromosomes, either or both of which could carry independently induced recessive lethal mutations. In 1996 as the F₃ cultures were being established, Barbara Wakimoto (Wakimoto *et al.* 2004) began testing the *cn bw* males for fertility (Table 1). Within a few generations of their establishment, ~10% of the lines lacked white-eyed flies, indicating that they had become fixed for a lethal-bearing *cn bw* chromosome and therefore could not be fertility tested. Eight years later Andrew Zelhof screened the collection for deep-pseudopupil abnormalities and found that the incidence of lethal fixation had increased substantially, to ~38% (A. Zelhof, unpublished data). The lethal lines found by Zelhof included many of those shown by Wakimoto *et al.* (2004), prior to fixation, to carry male-sterile mutations (Table 1).

With the goal of explaining the Zuker collection results, as well as increasing understanding of the processes of germline development and chemical mutagenesis transmission that underlie them, we developed and tested a model of gonadal mosaic development in *D. melanogaster*.

Two genetic end points have been used in earlier investigations of the origin and developmental fate of mutations chemically induced in mature sperm: specific locus and recessive lethal mutations. Each involves different experimental strategies. Specific-locus studies detect mutations of one or a few specific genes with easily scored adult phenotypes. Treated males are crossed to females homozygous for recessive alleles of genes being scored. Mutations are sought among the progeny as exceptions exhibiting mutant phenotype in some or all tissues. Chances of detecting somatic mosaics depend on the anatomical extent of expression of the locus being screened: *y* mutations expressed throughout the integument, *dp* expressed in thoracic structures, and *w* mutations affecting eyes. These are F₁ tests and, because of the low mutation rates of single genes, require large numbers of offspring of mutagenized males. The germline compositions of the mutants recovered can be assessed by test crossing. Recessive-lethal tests, on the other hand, score mutations at large numbers of vital loci simultaneously; they determine constitutions of gonads but not of the soma. Treated

chromosomes are recovered in heterozygotes and following a series of crosses are tested in homozygotes (or hemizygotes in the case of sex-linked recessives). Nearly all recessive-lethal experiments to date have involved sex-linked lethals. They are easy to score, but generally the lethal mutations cannot be complement-tested *inter se* and therefore cannot identify multiple mosaics. The experiments do enumerate gonadal mosaics, but they encounter replicating instabilities (Auerbach 1946a, b), i.e., repeat occurrences in later generations of mutations first encountered in the F₃. These instabilities are difficult to demonstrate in the case of autosomal lethals, are not understood, and are not dealt with in this communication.

Only Epler (1966) has scored for autosomal recessive lethals. From sons of EMS-treated males, he established 10 sublines balanced for the treated second and third chromosomes. In some cases where all the sublines were lethal, complementation tests established that the F₁ male was a double mosaic, containing two different second-chromosome lethal mutations. (For a tabulation of earlier experiments and their results see *Discussion*.) Epler's experiment had small sample sizes, in part because of the preexistence of a second-chromosome lethal mutation among the originally treated chromosomes, but clearly demonstrated the value of complementation in revealing the existence of multiple mosaics.

We repeated Epler's (1966) experiment on a larger scale to characterize more fully the genetic compositions of the germlines of the sons of mutagenized males. We also attempted to manipulate the ability of mismatch repair to assess the influence of repair on mutagenesis.

Materials and Methods

Crossing scheme

We fed 26 recently isogenized wild-type males (P₀ designated A–Z) on a 25 mM solution of EMS; this dose had been found by Aaron and Lee (1978) to produce 45% sex-linked-recessive-lethal mutations (Figure 1). Thirteen treated males (A–M) were crossed to *cn bw*; *e* females and 13 (N–Z) to mismatch-repair-deficient females [*mus201*^{Z1953} *cn bw*/Df(2L)ED623, *cn bw*; *e*]. This genotype was generated immediately prior to use in the P₁ by crossing *SM6b*, *cn bw*/*mus201 cn bw* to *SM6b*, *cn bw*/Df(2L)ED6223, *cn bw*. The resulting *mus*/Df genotype was shown to be mutagen sensitive by its failure to survive in parallel crosses raised on MMS supplemented medium. Laurencon *et al.* (2004) recovered *mus201*^{Z1953} from a screen of the Zuker collection. We chose the use of hemizygous females in this cross to avoid effects of any recessive modifiers that may have accumulated in the *mus201* stock. From each of these crosses, five F₁ males, each of which developed from an egg fertilized by a treated sperm, were selected for analysis.) To resolve mosaics and characterize the cellular contents of the germlines of the 130 resulting males, we crossed them individually to multiple females of the following constitution: *SM6b*, *Cy Roi cn*^{2P} *bw*/T(2;3)82F12, *cn bw*; *hid e*/TM6c, *Sb e*.

Table 1 Lethal fixation in the Zuker collection (Koundakjian et al. 2004) as a function of time

	Chromosome 2			Chromosome 3		
	Viable	Lethal	% lethal	Viable	Lethal	% lethal
Wakimoto et al. (2004) unpublished observation, 1996	5398	746	12.1	6104	365	5.64
Zelhof (unpublished results, 2006): total	3634	2240	38.1	3384	2035	37.55
Subset of stocks scored by Zelhof for lethality that had been scored as male sterile by Wakimoto	685	254	27.1	800	451	36.05

The data from 2006 include the total collection as it existed at that time (A. Zelhof, unpublished results).

(*hid* = $P\{hs-hid\}$ is a dominant temperature-sensitive lethal form of head involution defective.)

Backcross for purposes of mutation amplification

Ten to 12 F_2 $cn^+ bw^+ e^+$ males (designated a through j or l) were selected from each of these crosses. They were either *Cy Sb* (i.e., $SM6b/2^*$; $TM6c/3^*$) or phenotypically wild type (i.e., $2^*/T(2;3)82F12/3^*$). Note that these males carried descendants of a single mutagenized autosomal complement; they also carried a mutagenized Y chromosome and possibly a mutagenized 4th; they did not carry a mutagenized X, or the maternally derived *cn bw*, the *mus cn bw*, or the *e* chromosome present in the original cross.

Heat-shock generation to isolate males and females heterozygous for mutagenized autosomes

At this point of the crossing scheme we approached our goal of 1300 males bearing mutagenized autosomal complements isolated from 130 testes. However, to characterize these complements we needed to make them homozygous and therefore required females of the same constitution (i.e., heterozygous for the same autosomal complement) for inbreeding. Therefore the F_2 males were backcrossed to females of the maternal genotype. These F_3 crosses were treated in the larval stage with a 37° heat shock, killing off all $T(2;3)$ -containing offspring. The only surviving progeny were $SM6b/2^*$; $TM6c/3^*$.

Inbreeding generation to produce homozygotes for treated autosomes

When both F_3 males and females survived the heat shock, they could be inbred to produce F_4 homozygotes for the treated autosomes. Heat-shock survivors were few in number and sometimes failed to produce both males and females, postponing the possibility of making the required cross. In these cases, it was possible to recross the single-sex survivors of heat shock to the parental translocation-bearing flies and, following heat shock, to again select *Cy Sb* male and female heat-shock survivors in the next generation for inbreeding.

Results

The experiment began with 26 treated males (A–Z) each producing five sons (e.g., A1–A5 each representing an individual germline). Each of the five sons then produced 10–12 lines, each representing an individual germline cell from an F_1 male (Figure 2).

Genetic products of crosses

Four classes of offspring were produced from the sample crosses:

Curly Stubble ($Cy/2^*$; $Sb/3^*$),
Curly ($Cy/2^*$; $3^*/3^*$),
Stubble ($2^*/2^*$; $Sb/3^*$),
Wild type ($2^*/2^*$; $3^*/3^*$).

A sample of up to 12 crosses, characterizing the cellular contents of a germline could produce three classes of results: all crosses could fail to produce homozygotes, e.g., all lines could produce only *Cy* offspring, indicating a complete gonadal lethal on chromosome 2, designated *L*; all could produce viable homozygotes designated *P* (parental); or some could produce homozygotes in some crosses and not in others, i.e., the germline was mosaic, designated *M*. These alternatives obtain for both chromosomes 2 and 3. The relative frequencies of these outcomes are tabulated in Table 2. To account for the occasional escaper, we classified chromosomes producing <5% recovery as lethal. All males were fed mutagen together so that the dose to which they were exposed was the same, although the amount they ingested could vary. The crosses were designed to assess the role of repair in mutagenesis. Males from lines A–M were crossed to mismatch-repair-proficient females and those from lines N–Z were crossed to mismatch-repair-deficient females. The data are homogeneous with no discernable effect of crosses involving mismatch-repair-deficient *vis-à-vis*-proficient females, nor are results from chromosome 2 different from those from chromosome 3.

The absence of an effect of the mutagen-sensitive allele of *mus201* on the results indicates that we were unable to manipulate the probability of repair of mutations. Consequently, we conclude that the repair system remained intact throughout the experiment.

Testing to identify multiple mosaics in the lethal component of germlines containing lethal mutations

For the classes in which some or all of a line carried a lethal (*L* and *M*), we performed complementation tests to determine whether all the lethal-bearing chromosomes in a germline carried the same lethal mutation. Based upon the outcome of these tests, lethal components of observed classes were subdivided (see Table 3, columns labeled “Complementation”). For example, in the case of male H3, which was scored *L* for both chromosomes 2 and 3, we crossed *Cy Sb* females

Figure 1 Crossing scheme designed to make it possible to isolate and characterize the germ lines of the sons of EMS-treated males. *T(2;3)82F12* with *P[hs-hid]* was provided by Ruth Lehman.

from one line (H3b) to heterozygous males from each of the other H3 lines. In all such crosses the third chromosome was lethal, but in 5 of 11 the *trans*-heterozygotes were viable for chromosome 2. Accordingly, five lines were *l(2)a*; *l(3)* and seven were *l(2)b*; *l(3)*. We scored germline H3 as double mosaic (dm) for chromosome 2 and lethal for chromosome 3. We did not test for mosaics from lines in which the lethal component was too small (two or fewer samples). For germ lines A–Z, 70 of the 80 chromosomes 2 that were complementation tested were nonmosaic, 9 were double mosaic, and 1 was triple mosaic (*i.e.*, three different lethals); of the 87 chromosomes 3 that were complementation tested, 84 were nonmosaic, 2 were double mosaic, and 1 was triple mosaic.

Chi-square testing to identify multiple mosaics in the parental component of mosaic and parental germ lines

Double mosaics demonstrate that the male germline may contain two or three types of cells with respect to the lethal alleles that they carry on a particular autosome. We entertained the thought that germ lines may also contain more than one type of nonlethal chromosome. Homogeneity χ^2 tests showed significant heterogeneity in the survival rates of nonlethal homozygotes, both within and among germ lines, suggesting the presence of semilethal mutations (columns labeled "Probability" in Table 3). In some lines the survival rates were homogeneous, but in others they were wildly heterogeneous with infinitesimal probabilities that comprised a single population. In the latter cases we sorted the data according to survival rate and then divided the sample into two subsamples and measured their homogeneity. Table 4 illustrates the efficacy of this procedure and shows the consequences of subdividing the data using example cases in which the probabilities of homogeneity were $<1\%$. As an example, nine lines from germline E2 were nonlethal for chromosome two. The probability that they comprised a single population was $3.54E-06$; however, division into subsamples of four and five lines resulted in probabilities of 0.238 and 0.504 respectively. Thus we concluded that germline E2 was mosaic. Our procedure subdivided any line with $P < 0.05$ into subsets of greater probability and designated them

mosaic. A caveat here is that any set of heterogeneous data can be subdivided into more nearly homogeneous subsets, but the results in Table 4 appear to be striking enough not to be artifactual. In Table 4, lines A–M show eight cases with $P < 0.05$ including four with $P < 0.01$. Lines N–Z have nine lines with $P < 0.05$ including two with $P < 0.01$. In Table 4, the appearance of *M*, *dm* or *tm* (triple mosaic), or a probability <0.05 , in any row indicates the presence of gonadal mosaicism: 52% in A–M and 49% in N–Z were mosaic.

Modeling Germline Genesis

We adopted and made more explicit a model for formation of the germline during development and transmission to the gonads of the lesions produced by chemical alteration of the chromosomal DNA of the fertilizing sperm. We built upon the assumptions first proposed by Krieg (1963) in studying chemically induced mutation in bacteriophage and subsequently utilized by Lee *et al.* (1970) in characterizing somatic mosaics for *yellow* in *Drosophila*. They postulated that alkylated DNA generates mutations during phage replication (Krieg 1963) and during nuclear divisions leading to blastoderm formation (Lee *et al.* 1970).

Mutagenesis during syncytial development

Following fertilization, the zygotic nucleus embarks upon successive rounds of synchronous syncytial mitotic divisions. We postulate that the DNA strands of a spermatozoon that have been modified (*e.g.*, alkylated) by the mutagen are stable. The descendants of the daughter nucleus of each replication of an alkylated strand comprise a clone of genetically identical nuclei, the size of which depends on the stage of replication at which the clone is generated. At the genesis of each clone, the alkylated strand may misreplicate and produce a recognizable mutant clone.

The following discussion makes reference to the accompanying Microsoft Excel workbook *Modeling Gonadal Composition*. The workbook contains 16 worksheets: the sheet entitled *Generate Syncytium* randomly generates the clonal composition of the syncytium at the eighth division, the sheet entitled *Germline Selector* generates the results of 130

Table 2 Classifications of initial counts of the inbreeding generation

Germlines	Chromosome	<i>L</i>	<i>M</i>	<i>P</i>
A–M	2	39	10	14
A–M	3	42	8	12
	Σ	81	18	26
N–Z	2	31	14	15
N–Z	3	39	12	9
	Σ	70	26	24
	$\Sigma\Sigma$	151	44	50
		62%	18%	20%

Classifications are designated in *italic* as *L*, *M*, and *P*, where *L* denotes crosses that fail to produce homozygotes, *P* denotes crosses in which all produce viable homozygotes, and *M* where some do and some do not. All males were exposed to mutagen together. Males A–M were crossed to mismatch-repair-proficient females and males N–Z were crossed to mismatch-repair-deficient females.

Lee (1998) estimate two to four primordial germline nuclei from perusal of the literature. Gao *et al.* (2014) describe a very large experiment examining spontaneous autosomal lethal mutation rates using a crossing scheme that is coincidentally very similar to the one described in this communication; based on statistical analysis they propose that the number of primordial germline nuclei is greater than that reported in the literature. We have greatest faith in estimates based on direct observation of embryonic development. In particular we rely on the conclusions of Campos-Ortega and Hartenstein (1997) as the most recent and most explicit description of events following the eighth round of mitotic amplification of the zygote nucleus. Campos-Ortega and Hartenstein (1997) report that the three founding nuclei continue syncytial amplification for two more divisions in the polar plasm; the nuclei then move into polar buds, which undergo two further divisions, still in synchrony with blastoderm nuclei. Thus the polar plasm becomes occupied, not by the three founding nuclei, but by the products of their proliferation, which we believe may have led to overestimates of the numbers of founding nuclei. (We provide support for our assumption of three founding nuclei by comparing our experimental results to those predicted by the model while varying the number of modeled progenitor nuclei. For a discussion of this see [Supporting Information, File S1, File S2, and File S3.](#))

Identities of nuclei selected: The model generates a linear array of products in which adjacent nuclei tend to be correlated in origin (Sheet *Generate Syncytium*, columns AH:AJ, [File S3](#)). How does one choose three nuclei from this array to populate the germline? Simply selecting several adjacent nuclei as germline progenitors does not adequately sample nuclear diversity. On the other hand, choosing nuclei at random fails to reflect the nonrandom distribution of nuclei in the embryo. As has been observed in several studies of early development, the discernable products of early mitotic X chromosome loss give rise to contiguous domains of male nuclei in gynandromorphs and of ring-X chromosome loss, as demonstrated cytologically in syncytial-blastoderm nuclei (Zalokar *et al.* 1980). That is, the descendants of a single

mitotic division tend to remain proximate to each other during ensuing amplification. Accordingly, we view the disposition of nuclei following the eighth mitotic division in the syncytial blastoderm as a pattern of patches, as in a three-dimensional patchwork quilt, the sizes and arrangement of the patches reflecting the provenance of the nuclei. We propose that a fraction of this assemblage occupying the posterior region of the embryo is the source of the primordial germline nuclei.

To strike a compromise between a too random and insufficiently random selection of germline precursors, the model arranges the one-dimensional column of 256 nuclei into a logical ring, such that nuclei carrying Watson and Crick-derived strands are adjacent at two diametrically opposed points on the circumference of the ring. The model identifies 20 adjacent nuclei in this ring (*e.g.*, Sheet *Germline Selector*, cells L11:N69 [File S3](#)). (Note that how many adjacent nuclei are selected in the preceding step determines the randomness of the sample used in the following step. We tested selecting as many as 50 nuclei and found that it had virtually no effect on the model results). From each of these samples of 20 adjacent nuclei, one through eight are selected at random to be germline progenitors (*e.g.*, Sheet *Germline Selector*, cells L75:N97, [File S3](#)). These germline-progenitor nuclei may be derived from one to several clones, leading to monoclonal or polyclonal germlines. In the case of three nuclei being selected as progenitors, at least one nucleus carries a mutagenic strand ~5% of the time; when this happens, the model treats it as an additional contributing nucleus because it could continue to generate clones during later gonad development. In addition, the probability that descendants of both Watson and Crick will be included among the selected nuclei is between 8 and 9%. Accordingly the majority of mosaic germlines are derived from one of either the Watson or the Crick strand, not both.

These considerations also provide insight into the production of gonadal mosaics following X irradiation. Assume that X-ray-induced lesions occur at the level of the individual strands of the DNA, and that those lesions may be stable and amplified unchanged during development, producing mosaic embryos in which half of the nuclei contain descendants of the modified strand and half from the complementary strand. Assuming that the germline is derived from around three nuclei from the mosaic syncytium and following estimates from the preceding paragraph, all three may carry the same nuclear product, either mutant or nonmutant, thus producing nonmosaic germlines; alternatively 8–9% of the germlines carry descendants of both strands and will be mosaic.

Lethal mutagenesis: The likelihood of misreplication is a variable in the model (Sheet *Germline Selector*, cell F80). Based upon this variable, the model determines at random which clones bear a lethal mutation and which are parental. The model tabulates results for the cases in which one through eight nuclei are selected as progenitors (*e.g.*, Sheet *Germline Selector*, cells L100:N107, [File S3](#)). Here we use a notation of

Table 3 Scoring of the homozygosing generation

Mismatch repair proficient										Mismatch repair deficient									
Chromosome 2					Chromosome 3					Chromosome 2					Chromosome 3				
Line	Class	Complementation	Probability	Line	Class	Complementation	Probability	Line	Class	Complementation	Probability	Line	Class	Complementation	Probability	Line	Class	Complementation	Probability
A1	L			N1	P			N1	P		0.175	N1	P			N1	L		
A2	L			N2	L			N2	L			N2	L			N2	L		
A3	L			N3	M			N3	M			N3	M			N3	M		0.073
A4	L			N4	L		0.9	N4	L			N4	L			N4	L		
A5	P		0.08	N5	P		0.050	N5	L			N5	L			N5	L		
B1	L	dm		O1	M			O1	L			O1	L			O1	P		0.002
B2	L	dm		O2	P		1.6596E-05	O2	L			O2	L			O2	L		
B3	L			O3	L			O3	L			O3	L			O3	M		
B4	L			O4	L			O4	M		0.170	O4	M			O4	L		
B5	M	dm	0.121	O5	M	dm	1.77E-09	O5	L			O5	L			O5	L		
C1	L			P1	L			P1	M		0.394	P1	M			P1	M		0.395
C2	L			P3	L			P3	P		0.14	P3	P			P3	P		0.061
C3	L			P4	L			P4	M			P4	M			P4	M		
C4	L			P5	L			P5	L			P5	L			P5	L		
C5	L			Q1	L			Q1	P		0.638	Q1	P			Q1	L		
D2	P		0.911	Q2	P		0.242	Q2	L			Q2	L			Q2	L		
D3	P		0.113	Q3	P		0.55	Q3	L			Q3	L			Q3	P		
D4	L			Q4	L			Q4	M			Q4	M			Q4	M		
D5	L			Q5	L			Q5	M			Q5	M			Q5	P		0.692
E1	P		3.54E-06	R1	L			R1	M		0.044	R1	M			R1	P		0.156
E2	P		0.702	R2	P		0.226	R2	L			R2	L			R2	P		0.024
E3	L			R3	L			R3	P			R3	P			R3	M		
E4	M		0.095	R4	L			R4	L			R4	L			R4	M		0.808
E5	L			R5	P		0.011	R5	L			R5	L			R5	L		
F1	L			S1	L			S1	M		1.73E-05	S1	M			S1	M		0.0001
F2	M		0.011	S2	L			S2	M		0.025	S2	M			S2	L		
F3	P		0.0002	S3	L			S3	P		0.075	S3	P			S3	L		
F4	L			S4	L			S4	L			S4	L			S4	L		
F5	M		0.0003	S5	L			S5	M			S5	M			S5	L		
G1	L			T1	P		0.193	T1	L			T1	L			T1	P		3.79E-06
G2	L			T2	L			T2	P			T2	P			T2	M		0.005
G3	L	tm		T3	L	tm		T3	P		1.12E-07	T3	P			T3	L		
G4	L			T5	P		0.815	T5	P		0.026	T5	P			T5	L		
G5	L			U1	L			U1	L		0.296	U1	L			U1	L		
H1	P		0.324	U2	L			U2	P		0.246	U2	P			U2	L		
H2	L			U3	L			U3	M		0.109	U3	M			U3	L		
H3	L	dm		U4	L			U4	L			U4	L			U4	M		0.393
H4	P		0.146	U5	L			U5	L			U5	L			U5	L		
H5	L			V2	L			V2	P		0.847	V2	P			V2	L		
I1	P		0.341	V3	L			V3	L			V3	L			V3	L		
I2	P		0.821	V5	P		0.903	V5	L			V5	L			V5	L		
I3	M		0.162	W2	M		0.0008	W2	M		0.011	W2	M			W2	L		
I4	M		0.606	W3	P		0.107	W3	L			W3	L			W3	L		

(continued)

Table 4 Data showing survival probabilities when subdividing nonhomogenous lines

Line	Chromosome 2 or 3	Probability	Component probabilities			Class	Ratio of components
B2	3	1.66E-05	0.231	0.976	0.469	<i>P</i>	8:2:1
B5	3	1.77E-09	0.486	0.474			5:4:3
E2	2	3.54E-06	0.504	0.238		<i>P</i>	5:4
E4	2	1.01E-12	0.374	0.221		<i>M</i>	4:2
F3	2	0.0002	0.259	0.509			6:5
F5	2	0.0003	0.869	0.311		<i>P</i>	3:4
I3	3	0.0008	0.205	0.344		<i>M</i>	2:2
J4	2	3.14E-06	0.912	0.292		<i>P</i>	8:3
J4	3	6.99E-06	0.278	0.349		<i>M</i>	7:4
L4	3	0.002	0.719	0.52		<i>P</i>	6:4
O1	3	0.002	0.593	0.171		<i>P</i>	5:2
K3	3	8.76E-10	0.543	0.27		<i>M</i>	82
S1	2	1.70E-05	0.33	0.168		<i>M</i>	2:5
S1	3	0.0001	0.667	0.099		<i>P</i>	7:3
S5	3	3.79E-06	0.7	0.723		<i>P</i>	7:4
T2	2	1.12E-07	0.895	0.605		<i>P</i>	6:4:2
Z2	3	0.001	0.431	0.605		<i>M</i>	6:5

Blank spaces indicate components represented by a single cross for which no probability can be calculated.

L to denote a chromosome with a lethal mutation and *P* to denote parental. When multiple nuclei are selected as germline progenitors, the model repeats *L* or *P* to denote variation. For instance, *LL* denotes a double lethal mosaic (see Table 5).

Monte Carlo analysis: Sheets *Germline Selection 1* through *Germline Selection 10* store 130 independently generated sets of gonadal progenitors (e.g., Sheet *Germline Selection 1*, L75: YI97, File S3). The results when one through eight nuclei are selected as progenitors are tabulated (e.g., Sheet *Germline Selection 1*, J215:CZ344, File S3) using the terminology listed in Table 2 (*L* for complete gonadal lethal, *P* or parental for viable homozygotes, and *M* for mosaic germlines). The clonal compositions of these classes are outlined for three precursor nuclei in Table 5.

Finally, we ran the entire model 100 times for mutation rates 0–1 in increments of 0.05 (Sheet *Final Monte Carlo Results*, File S3). This resulted in 336,000 rows of model results [(100 model runs) × (10 syncytium simulations) × (21 mutation rates from 0 through 1) × (2 chromosomes) × (8 different progenitor nuclei counts of 1 through 8) = 336,000].

Some investigators have reported effects that are not accounted for in this model: mutations that emerge three or more generations after exposure to EMS, which they attribute to replicating instabilities (Auerbach 1946a,b; Mathew 1964), or complete mutations in the *F*₁. Our model does not accommodate complete mutations (i.e., those that affect the whole soma) but may be appended to do so (see *Discussion*).

Reconciliation of the Data with the Model

Composition of *L*, *M*, and *P*

At this stage in the analysis of the experimental results we knew that the germlines of sons designated *P* contain no lethal mutations, that germlines designated *M* contain a mix of lethal and parental cells, and that germlines designated *L* do not contain any parental cells. But this was incomplete—for instance,

germlines designated *L* could contain more than one lethal chromosome type. Similarly, lines designated *P* could contain semilethal chromosome types, and lines designated *M* could be heterogeneous for both lethal and parental components. We therefore conducted further analysis to uncover the presence of double and triple mosaic gonads in sons of treated males. Table 6 tabulates the genotypes of the germ cells sampled from each germline. According to the model, the alkylated strands of chromosome 3 descend through the eight mitotic divisions independently of those of chromosome 2. Thus the constitutions of the two chromosomes are not correlated. However, it is necessary to make the chromosomal constitution of separate germline cells consistent. Originally Table 6 listed the constitutions of chromosomes 2 and 3 separately; however, each germline cell contained both chromosomes, so we attempted to reconcile these disparate observations into a single set of germline-cell constitutions. For example, Line Z2 segregated for lethals on both chromosomes. Chromosome 2 was homogeneous for survival at 38.2%. The survival of chromosome 3 was highly heterogeneous with a probability of <0.1%; removing Z2i, which is lethal, increases the probability to 1.0%, which is still highly heterogeneous. The remaining third chromosome lines of Z2 can be subdivided into sublines *khfe*, *P* = 0.964 and *dbjcg*, *P* = 0.249. The chromosome 3 results indicate that the germline contains three different genotypes; i.e., chromosome 2 is monoclonal and chromosome 3 is polyclonal in origin. We next apportion the chromosome 2 data into these three classes, determining the survival of each as illustrated in Table 6. We used this method for all similar cases.

Table 6 details the numbers and survival of different classes of offspring. Survival <5% was classified as lethal; double mosaic lethals were designated a and b. The remainder (survival >5%) were tested for homogeneity, and those with probabilities <0.05 were subdivided and entered separately; homogeneous classes are set in italic type. The model does not predict overlapping clones of chromosomes two and

Table 5 Possible origins and clonal compositions of germlines labeled *L*, *MP*, and *P* in the case where 3 nuclei are selected as germline progenitors

Initial classification	Possible germline compositions for three precursor nuclei				
	Premitotic: Complete mutations (whole body)	Mitotic			
		Monoclonal	Polyclonal		
		Single	Double mosaics	Triple mosaics	Quadruple mosaics
<i>L</i>	<i>L</i>	<i>L</i>	<i>LL</i>	<i>LLL</i>	<i>LLLL</i>
<i>M</i>			<i>LP</i>	<i>LPP, LLP</i>	<i>LPPP, LLPP, LLLP</i>
<i>P</i>	<i>P</i>	<i>P</i>	<i>PP</i>	<i>PPP</i>	<i>PPPP</i>

Each roman *L* denotes a lethal-bearing chromosome present in the germline and *P* denotes a nonlethal or parental chromosome. A monoclonal germline descends from a single clone, which may be either lethal or parental. Diclinal germlines descend from two clones, which could carry complementing lethal mutations (*LL*) or two clones without lethal mutations (*PP*) with different survival rates or one with and one without a lethal mutation (*LP*). A germline derived from as many as four different clones is a possible outcome even when only 3 nuclei are selected, as a result of the cases in which an alkylated strand is present in one of the progenitor nuclei.

three; they may be coincident or included one within the other, but it is never the case that a clone for one chromosome can be spread across more than one clone of the other chromosome. Three cases of apparently overlapping clones, J4, S5, and T2, are found in Table 6. We have reconciled these clones with the model by postulating that a single alkylation caused the same misreplication in separate nuclear divisions. We indicate this with boldface type and by subdividing the overlapping clone into two clones with boxes. This suffices for S5 and T2 but not for J4. Because the mutation rate per alkylation is so low, it is unsatisfying to accept repeat mutations.

From the data as parsed in Table 6 it is possible to determine the degree of mosaicism and count the number of clones from which the germline is derived and the number of chromosomes from each clone that contribute to the next generation. Figure 3 captures the degree of mosaicism in each germline—i.e., the number of genotypes present. Table 7 captures lethal clone frequencies and lethal chromosome frequencies for chromosome 2 and 3 in the germline.

Taking line E1 in Table 6 as an example, the germline of line E1 contained cells with two different genotypes (one of which is *cdfih* and the other *ebjg*). The number of genotypes defines the degree of mosaicism. The germline for E1 contained three clones, two for chromosome 2 and one from chromosome 3. The clones contributed a total of 18 chromosomes to found the next generation: two clones contributed second chromosomes (5 from *cdfih* and 4 from *ebjg*) and 9 for chromosome 3 (all 9 from one clone, *cdfihebjg*).

We found no effect of crossing the treated males to mismatch-repair-deficient females. There are relatively few records of effects of *mus201* on mutation rates; they mostly examine complete mutations and use mutagens different from those used here. However, Vogel *et al.* (1985) report a 7.3-fold increase in the sex-linked-recessive-lethal mutation rate in MMS-treated males and 2.0-fold increase in EMS-treated males crossed to *mus201^{D1}* females. The earlier experiments used *D1* and *G1* alleles of *mus201*, whereas our experiment utilized *mus201^{Z1953}*. This allele was recovered from the Zuker collection of heavily mutagenized autosomes by Laurencon *et al.* (2004) by screening for chromosomes

unable to develop as homozygotes on medium supplemented with MMS and demonstrating allelism with *mus201^{D1}* by the same criterion. Data tabulated in Table S3 and Table S4 provide ratios of 1.25 for vermillion mutations and for sex-linked recessive lethals ratios of 9.6 and 5.9 for ethylenimine and 3.0 for methyl methane sulfonate-treated males crossed to *mus201^{G1}* vs. wild-type females, respectively. These data indicate that in the present experiments, the existence of an active repair system results in a lower mutation rate than what would have been observed had we been able to interfere with its function.

Application of the model

Model results: The model predicts a ratio of *L*, *M*, and *P* for mutation rates from 0 to 1. Figure 4 presents the results of running the model 100 times for mutation rates 0 through 1 in increments of 0.05 for the cases when one, two, three, or four nuclei are selected. Chance events during development introduce wide variation in model results. These events include the random paths of the independent alkylated strands in the generation of the syncytium, semirandom selection of germline progenitors, and random mutation events. As mutation rates increase, the frequency of *L* (red) in the results trends upward from 0 to 100% and the frequency of *P* (blue) declines from 100 to 0 over the same range. The rate of gonadal mosaicism *M* (green) increases as a function of the number of nuclei selected as germline progenitors and peaks at a mutation rate of 0.5. As a result of the occasional inclusion of an alkylated strand, mosaicism is nonzero even when only one nucleus is selected as progenitor.

Experimental results consistent with model prediction: It is not necessary to take recourse in any mechanism other than those described in the model to account for the experimental results. They were consistent with those predicted by the three-nucleus model for a 0.72 mutation rate (0.72 summed over 3000 autosomal vital genes averages to a mutation rate of 2.3×10^{-4} /gene) (Figure 5).

Estimating the frequency of complete (whole body) mutations: The model is based on the supposition that all

Table 6 Classification of individual germlines according to genotypes of different germline cells

Line	Germ cells	Survival		Line	Germ cells	Survival	
		Ch 2	Ch 3			Ch 2	Ch 3
A1	abdefgjkl	I(2)	I(3)	N1	abcdefghijkl	0.321	I(3)
A2	abdefghij	I(2)	I(3)	N2	af	I(2)	I(3)
A3	abcdefghij	I(2)	0.053	N3	dejkhi lg	I(2) I(2)	I(3) 0.095
A4	abcdefghijk	I(2)	I(3)	N4	acdefi	I(2)	I(3)
A5	abcdefghijl	0.275	0.294	N5	dfhl l	I(2) I(2)	I(3) 0.085
B1	abdfgijkl h c	I(2) I(2) I(2)	I(3)a I(3)b 0.265	O1	abcefgkl cd	I(2) I(2)	0.25 0.387
B2	abcdghil ef k	I(2) I(2) I(2)	0.214 0.382 0.066	O2	ab	I(2)	I(3)
B3	abcdefghijkl	I(2)	I(3)	O3	a f	I(2) I(2)	I(3) 0.23
B4	abcdefghijkl	I(2)	I(3)	O4	acdjkl	0.374	I(3)
B5	cg fil d bhlj ea	I(2)a I(2)b 0.354 0.333 0.284	I(3)a I(3)b I(3)c 0.098 0.313	O5	bk	I(2)	I(3)
C1	abcdefghijk	I(2)	I(3)	P1	elafij j	0.295 I(2)	0.323 I(3)
C2	abcefhj k	I(2) I(2)	I(3)a I(3)b	P3	fkI	0.162	0.137
C3	abcdefghik	I(2)	I(3)	P4	a l	0.228 I(2)	I(3) I(3)
C4	abcdghijk	I(2)	I(3)	P5	abghijkl	I(2)	I(3)
C5	abceffgh	I(2)	I(3)	Q1	abcdefghijkl	0.262	I(3)
D2	abcdefghij	0.276	0.306	Q2	h	I(2)	I(3)
D3	afcdefhijk	0.16	0.29	Q3	f	I(2)	0.134
D4	abcdefhijkl g	I(2) 0.26	I(3) 0.21	Q4	cegijk	I(2)	0.105
D5	defghk	I(2)	I(3)	Q5	adghil f	I(2) 0.75	0.22 0.05
E1	cdfih ebjg	0.084 0.23	I(3) I(3)	R1	fg abcdijkl b	I(2) 0.288 0.133	0.32 0.283 I(3)
E2	abcdefgij h	0.336 0.409	0.276 0.41	R2	adk l	I(2) I(2)	0.258 0.476
E3	abcdefghijkl	I(2)	I(3)	R3	ael j	0.059 0.306	I(3)a I(3)b
E4	acdgilhfjk be	I(2) 0.25	I(3)a I(3)b	R4	bald ij	I(2) I(2)	0.104 I(3)
E5	igce khbdjf	I(2) I(2)	0.297 0.149	R5	achjkl	I(2)	I(3)
F1	abcdefgi	I(2)	I(3)	S2	abcdefghijkl	I(2)	I(3)
				S3	abcdeghijkl		I(3)

(continued)

Table 6, continued

Line	Germ cells	Survival		Line	Germ cells	Survival	
		Ch 2	Ch 3			Ch 2	Ch 3
F2	acdeghi fj b	l(2) 0.292 0.522	l(3) l(3) l(3)	S4	acdefhikl j	l(2) l(2)	l(3)a l(3)b
F3	dgbh eafjil	0.453 0.3	l(3) l(3)	S5	gbcfike had j	l(2) l(2) 0.357	0.099 0.24 0.302
F4	abcdefghijkl	l(2)	l(3)	T1	cdefghij a b	l(2) l(2) l(2)	l(3) 0.131 0.302
F5	eha bfji	0.097 0.311	l(3) l(3)	T2	ed hcgf j k bi a	0.221 0.236 0.325 0.344 0.45 0.667	l(3)a l(3)b l(3)b 0.272 l(3)b l(3)a
G1	acdefghikl	l(2)	0.273	T3	ghfae jckbi	0.132 0.286	l(3) l(3)
G2	abcddefghijkl	l(2)	l(3)	T5	bcdefghijkl	0.371	l(3)
G3	abdfghijkl c e	l(2)a l(2)b l(2)c	l(3)a l(3)b l(3)c	U1	abcdfghijkl	l(2)	l(3)
G4	abcddefghijkl	l(2)	0.307	U2	adfgghijkl	0.33444	l(3)
G5	abcddefghi	l(2)	l(3)	U3	bcdj ei	0.047 0.32	l(3) l(3)
H1	abcddefghijk	0.3	l(3)	U4	abcefghijk	l(2)	l(3)
H2	abcefghijkl	l(2)	l(3)	U5	abcddefghijkl	l(2)	l(3)
H3	aegij bcdfhkl	l(2) l(2)	l(3)a l(3)b	V1	ceijkl	l(2)	l(3)
H4	abcddefghijk	0.3	l(3)	V2	abcefghijkl	0.3341	l(3)
H5	abcddefghijkl	l(2)	l(3)	V3	abcddefghijkl	l(2)	l(3)
I1	bcdegah	0.335	l(3)	V5	abcdefg	l(2)	l(3)
I2	gkhlfdeji ca	0.359 0.509	0.328 0.33	W2	ibhejag k d	0.106 0.333 l(2)	l(3) l(3) l(3)
I3	afhijl bc gk	l(2) 0.082 0.056	l(3) 0.126 0.213	W3	abehik	l(2)	l(3)
I4	bhi jklegcfa	l(2) 0.308	0.303 0.271	W4	jhlcvcd akf	l(2) l(2)	0.201 0.359
I5	abcdh kejif	l(2) 0.392	0.339 l(3)	W5	bcdefghijkl	l(2)	l(3)
J1	abcdghijkl f	0.35 0.632	l(3) l(3)	X1	acdefghijkl	l(2)	l(3)
J2	akfeijhdc g	0.355 l(2)	0.282 0.212	X2	cdefgj ik	l(2) 0.318	l(3) l(3)
J3	bcdefghijkl	l(2)	0.344	X3	fg ade bcijk l	0.323 l(2)a l(2)b l(2)b	l(3)a l(3)b 0.269 0.5
J4	kf i hab cedjg	0.621 0.5 0.326 0.324	l(3) 0.16 0.113 l(3)	X4	cabieg	0.325	l(3)
J5	abcddefghijl k	0.36 0.607	l(3) l(3)	X5	cdefghijk	l(2)	l(3)
K1	ifdgkaebj	0.302	l(3)				

(continued)

Table 6, continued

Line	Germ cells	Survival		Line	Germ cells	Survival	
		Ch 2	Ch 3			Ch 2	Ch 3
	c	<i>l</i> (2)	<i>l</i> (3)				
	h	0.493	<i>l</i> (3)	Y1	abcdefgij	<i>l</i> (2)	<i>l</i> (3)
K2	cdefghijkl	<i>l</i> (2)	<i>l</i> (3)	Y2	abcdefghijkl	<i>l</i> (2)	<i>l</i> (3)
K3	abcefgkl	<i>l</i> (2)	<i>l</i> (3)	Y3	abcfghij	0.342	<i>l</i> (3)
	c	<i>l</i> (2)	0.104				
	d	0.469	0.543	Y4	abcefgghik	0.248	<i>l</i> (3)
K4	behkl	<i>l</i> (2)	<i>l</i> (3)	Y5	abcdeghjk	<i>l</i> (2)a	<i>l</i> (3)
					f	<i>l</i> (2)b	<i>l</i> (3)
K5	cde	<i>l</i> (2)	0.188				
L1	bcdefghijkl	<i>l</i> (2)	<i>l</i> (3)	Z1	idajbcgf	0.081	<i>l</i> (3)
L2	abcdeghij	<i>l</i> (2)	<i>l</i> (3)		eh	<i>l</i> (2)	<i>l</i> (3)
				Z2	khfe	0.395	0.141
L3	acd hj	<i>l</i> (2)	<i>l</i> (3)		dbjcga	0.373	0.281
	gf	0.301	<i>l</i> (3)		i	0.414	<i>l</i> (3)
				Z3	abcdehj	<i>l</i> (2)	<i>l</i> (3)
L4	bcfgjk	<i>l</i> (2)	0.096				
	lhia	<i>l</i> (2)	0.202	Z4	abcdeghijkl	<i>l</i> (2)	<i>l</i> (3)
L5	bcdegijk	<i>l</i> (2)	<i>l</i> (3)	Z5	hibdf	0.234	<i>l</i> (3)
					iagkcje	0.388	<i>l</i> (3)
M1	abcdeghijkl	<i>l</i> (2)	<i>l</i> (3)				
M2	abcdeghijkl	<i>l</i> (2)	<i>l</i> (3)				
M3	abceghijkl	<i>l</i> (2)	<i>l</i> (3)				
M4	djk	<i>l</i> (2)a	<i>l</i> (3)				
	ehi	<i>l</i> (2)b	<i>l</i> (3)				
M5	acdeghijkl	<i>l</i> (2)	<i>l</i> (3)				

Survival of <5% was classified as lethal. Lethals are indicated by the lowercase letter *l*; double mosaic lethals were designated a and b, as in line C2 chromosome 3. The remainder (survival >5%) were tested for homogeneity, and those with probabilities <0.05 were subdivided and entered separately; homogeneous lethal classes are set in italic type and represent a single clone as in line B1 chromosome 2. Homogeneous nonlethal classes are also set in italic type and represent a single clone as in line E2 chromosome 2. Bold text indicates germ lines with overlapping clones.

lethal mutations take place at the level of the individual strands of the double helix, leading to the production of mosaic embryos. As developed, it does not predict the occurrence of complete (whole-body and thus whole-germline) mutations, such as those that have been observed in specific-locus mutation studies. In recessive-lethal mutation experiments, the treated chromosomes are retained in balanced heterozygous condition, and somatic constitutions cannot be distinguished. To account for complete mutations, we postulate that mismatches produced by some alkylations of the sperm DNA are resolved immediately upon fertilization (that is, before the joining of the male and female pronuclei), restoring complementarity in a new configuration and resulting in a complete mutation. Other alkylations, not resolved immediately, can misreplicate during the ensuing mitotic divisions giving rise to mosaic embryos. We refer to these two alternatives as premitotic and mitotic mutations (Table 5). What accounts for this difference in outcomes of alkylation is unexplained. The model as developed considers only mitotic mutations. However, the class designated *L* in the experiment likely included mutant products of both classes of alkylation. These two products cannot be differentiated experimentally.

Repeatedly running the model, using an assumption of three primordial germline nuclei, resulted in ~62% of gonads

derived from a single clone (monoclonal) and the polyclonal remainder derived from as many as three clones. Summing the experimental data we found 74% monoclonal germlines. The excess of monoclonals in comparison with expectations from the model we attribute to premitotic lethal mutations. Thus, in the experimental data, 12% *L* were derived from premitotic mutations and the remainder derived from mosaic embryos. Comparing the experimental result to the model after reducing *L* by 12% raised the percentage of *M* and *P*. This lowered the estimated mutation rate by several points (from ~0.72 to ~0.68).

Population of the gonads with stem cells: We entertained the possibility that transmissible instabilities could be the result of alkylated strands entering the germline and being passed on to the next generation. To explore this we describe the events leading to population of the presumptive gonad with germline stem cells. At the time of germline progenitor selection, when development is still under maternal genetic control, the constitution of the future germline is constrained by the constitution of the small numbers of nuclei sampled from the blastoderm as germline progenitors. Following polar bud formation, the polar buds cellularize, becoming pole cells; during gastrulation, when the zygotic genome takes control and the genotype of the zygote determines whether ovaries

Figure 3 The number of germ-cell genotypes (*i.e.*, clones) represented in each germline.

or testes will develop, the pole cells migrate anterodorsally. They proliferate as they migrate into the posterior midgut. Pole cells, 37–50, accumulate in the posterior midgut. Their class distribution will reflect that of the primordial germline nuclei. From the posterior midgut, samples of 10–15 cells migrate into the bilateral gonad primordia. This number approximates the number of germ cells in the hubs, the germline stem-cell niche, at the tips of the testes (Hardy *et al.* 1979).

We modeled these events using the worksheet *Germline Selection* for the case in which three nuclei are selected as germline progenitors, [File S3](#). Multiple runs of the program revealed that in ~10% of the presumptive germ lines, one of the three nuclei carries an alkylated strand. Assuming four rounds of mitotic pole-cell division during gastrulation, we postulated 48 descendants of the three primordial germline cells. Among the 48 pole cells postulated to populate the posterior midgut, one cell with an alkylated strand will be found in 10% of the embryos; 90% will contain no alkylated strands. The probability that the cell with a single alkylated

strand will be included within the sample of ~14 pole cells that migrate into the gonadal primordia is 0.3. Thus, the probability of a gonad receiving an alkylated chromosome is $0.1 \times 0.3 = 3\%$. The stem-cell niche of *Drosophila* males comprises 5–9 germline stem cells per testis (Hardy *et al.* 1979); assuming 14 total stem cells per male, 3% of males will contain one testicular stem cell with an alkylated strand. We had considered the possibility that this might account for replicating instabilities; however, in the case where an alkylated strand was transmitted from the stem cell to a gonoblast, it would then undergo four mitotic followed by two meiotic divisions to produce 64 spermatozoa, only 1 of which could carry the mutagenic strand: That would be one alkylated spermatozoon among the thousands of sperm produced by the testis. Therefore, the likelihood of transmission of the mutagenic strand to future generations seems infinitesimal.

This observation is incompatible with the notion that transmission of the alkylated strand can be passed to the next generation, much less through multiple generations, and therefore cannot account for the reappearance of new mutations in

Table 7 Frequency of lethal clones and chromosomes present in each germline

Clones	Chromosome 2			Chromosome 3		
	Parental	Lethal	% lethal	Parental	Lethal	% lethal
A–M (<i>mus+</i>)	37	52	58.43	30	57	65.52
N–Z (<i>mus-</i>)	33	44	57.14	25	54	68.35
Chromosomes	Parental	Lethal	% lethal	Parental	Lethal	% lethal
A–M (<i>mus+</i>)	194	433	69.06	164	456	73.55
N–Z (<i>mus-</i>)	188	294	61.00	99	386	79.59

Figure 4 Results of running the model 100 times for mutation rates 0 through 1 in increments of 0.05 for the cases when one, two, three, or four nuclei are selected as germline progenitors.

Figure 5 Comparison to three-nuclei model results of experimental results using combined proficient and deficient data ($L = 0.62$, $P = 0.20$, $M = 0.18$).

the generations that follow. Thus we rely on the formerly postulated transmissible instabilities (Auerbach 1946a,b; Mathew 1964) to explain the reappearance of a previously recovered mutation. The molecular nature of these delayed or repeated mutations is unknown and is worthy of further investigation.

Discussion

Zuker collection reassessed in light of the model

The experiments reported in this communication were undertaken to resolve confusion originally caused by the delayed fixation of lethals in lines judged to be lethal free in the Zuker collection. We applied the generalizations outlined above to interpret the outcomes of the screen used to generate the collection (Koundakjian *et al.* 2004). They reported that 84% of the autosomes tested were lethal. The lethals were descendants of F_1 males with premitotic lethal mutations and of males with monoclonal germlines from mitotic lethal mutations. The high incidence of lethal mutation is attributable to the massive doses of EMS employed and predicts a high incidence of mitotic lethal mutations among the clones sampled in polyclonal germlines. Males with polyclonal germlines will produce mosaic progeny of several constitutions, which on inbreeding may generate surviving genotypes. These survivors may carry nonlethal as well as lethal-bearing chromosomes, which may include complementing lethals. The survivors will be white eyed: *cn bw* for chromosome 2 and *bw st* for chromosome 3. Therefore, survivors were classified as lethal-free homozygotes despite the fact that they were often of mixed genotype and could segregate for lethal alleles in future

generations. In effect, the screen selected for mosaics, which, although appearing to be lethal free, were actually being saved in stocks of mixed genotypes that could become fixed for lethals in subsequent generations. Males with premitotic viable mutations were included among the surviving white-eyed offspring and expressed the mutant phenotype. However, the chromosomes with premitotic mutations had to run the gauntlet of eight rounds of mitotic replication of the alkylated chromosome before incorporation into the germline. Thus germlines were homogeneous for premitotic mutations as well as mutations in monoclonal germlines. When the latter mutations were recessive lethals, these lines accounted for the 84% recessive lethal mutations discarded at the beginning of the screen. Polyclonal germlines resulting from mitotic replication are gonadal mosaics, which may be heterogeneous for mitotic mutations. Accordingly, mosaic products from mitotic mutations will remain heterogeneous, containing both lethal and viable mutations as well as non-mutant chromosomes. These lines can be effectively screened for premitotic mutations as long as they are tested before lethal fixation (see discussion of male-sterile mutations below).

Within a few generations of establishing stocks, some lines of the Zuker collection had become fixed for a lethal mutation, demonstrating that the line was a gonadal mosaic for the lethal. Lethal fixation continued as the stocks were maintained by transferring, and 8 years later, Zelhof observed that almost 40% of lines had become lethal. Presuming that by that time lethal fixation had gone to completion, it stands to reason that other components of the mosaic gonads had likewise become fixed, resolving the problems of mosaicism for screening for viable mutations.

The observation of Wakimoto *et al.* (2004) suggesting that ~2000 of 12,000 Zuker lines tested were heterozygous for male-sterile mutations is consistent with the foregoing treatment of lethal mutations. These male-sterile mutations were presumably premitotic and/or mitotic monoclonal in origin; those that came from polyclonal germlines from premitotic-mutant-bearing sperm subsequently became fixed for mitotic lethal mutations (Table 1).

The Zuker stocks were also screened by Tilling for new mismatch mutation in exon sequences of specific genes (Till *et al.* 2003). The procedure, however, does not depend on homozygous survival of the chromosome being tested; the presence of mismatch mutations is revealed both in heterozygotes and mixed cultures. Isolating the mutation from such a mixed culture is a challenge. In retrospect, for Tilling it was unnecessary to go to the trouble of trying to select lines homozygous for lethal-free autosomes. A more recent collection generated by Scott Hawley (unpublished results) as a platform for Tilling, stocked mutagenized autosomes in balanced heterozygotes.

Considerations for forward genetic screens

The lessons for forward genetic screens are quite straightforward: One should not assume that the rate of gonadal mosaicism decreases with dose. In fact, although the rate at which the germline contains both lethal and parental nuclei does decrease, the rate of double and triple lethal mosaics increases in a nearly linear fashion in response to dose. So regardless of dose it is important to follow the standard procedure of carrying the crosses for an extra generation in order to sample single germ cells from the offspring of the treated male.

Implications for human health

If, as we propose, modified DNA molecules are stable, and mutagenic, then there are implications for human health. As we are frequently exposed to various types and levels of chemical insult, we may accumulate within our somatic cells modified DNA molecules that are misreplication prone and may act as mutagenic time bombs. Although modification may be at a low level, the number of opportunities is great. This may have implications for treatments of cancer, because treatments that eliminate pathologic cells may leave behind mutagenic cells that can generate the same mutation anew. While there is reason to suspect that corruption of stem cells can lead to cancer, the current view regarding the mechanism is that such cells have mutations in genes that lead them or their progeny to respond improperly to growth-regulating signals. The alternative suggestion that instead such cells may harbor a persisting modified and mutagenic strand of DNA that with some frequency continues to spawn the same mutation would require a different type of strategy for combatting such cancers since it would not suffice to eliminate all cells carrying the causative mutation. One would also need to find and eliminate the stem cell carrying the mutagenic strand, which would have no other obvious distinguishing feature.

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GENETICS

Supporting Information

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Gonadal Mosaicism Induced by Chemical Treatment of Sperm in *Drosophila melanogaster*

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File S1

Support for Assumption of 2 to 4 Germ-Line Progenitor Nuclei

There is some contention in the literature regarding the number of progenitor nuclei that found the germline. Estimates range from 2 through more than 8. We have tested these postulates by comparing the clonal composition of germ lines in our observed data to model results while varying several parameters in the model. The term “clone” is used here to denote all the descendants of one replication of an alkylated strand. Clonal composition is defined as number of different clones represented in the germ line. To make this comparison we first eliminated several experimental lines that had resulted in overlapping clones and were therefore ambiguous. (See Table 6 in the body of the main paper – the excluded lines are denoted in red.) We summed results of data from chromosome 2 and 3 three. We removed 12% from the observed the number of monoclonals to account for complete mutations as described in the body. (see discussion of the contribution of completes in the main texts in the section entitled *Estimating the frequency of complete (whole body) mutations*).

As a result of stochastic n events leading up to the selection of germ-line-progenitor nuclei, our model predicts a distribution in the ratio of mono- and poly-clonal germ lines. By running the model repeatedly while varying the degree of randomness with which the nuclei are selected from the syncytium, and by varying the number of progenitor nuclei, we are able to confirm that the best fit of the model parameters to our experimental data lies between two and four progenitor nuclei.

We vary the degree of randomness with which nuclei are selected by varying the size of the pool of nuclei from which progenitors are drawn from 20 through 50 (the smaller the pool, the more closely related the nuclei). Varying the pool size from 20 to 50 nuclei had little effect. By varying the number of progenitor nuclei from 1 through 8 we find that the model configured for one progenitor nucleus predicts more monoclonals and fewer polyclonals than observed. Models configured for four through eight progenitor nuclei predict too few monoclonal, and an excess polyclonals above tri-clonal. The model configured for three progenitor nuclei fits the observed data for every clonal count with the exception of five: the model predicts that there will be no nuclei with a clonal count of five and the observed data contain a single case. This one case may be an experimental artifact, or the result the inclusion of an alkylated strand that then amplified after germ line selection, the affect of which not entirely taken into account by the model. Regardless, a complete fit of the model parameters to the observed data is obtained by the reasonable assumption that the observed data result from germ lines descended from a mix of two, three or four progenitor nuclei.

File S1

Table S1 Comparison of model predictions to observed clonal counts assuming one through eight progenitor nuclei.

		Number of Progenitor Nuclei																
			1		2		3		4		5		6		7		8	
Pool	20	Observed	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Clonal Counts	1	71.8%	97.3%	100.0%	59.8%	79.7%	45.7%	72.4%	41.7%	66.9%	38.1%	60.2%	36.9%	60.2%	34.9%	57.1%	32.9%	54.3%
	2	20.9%	0.0%	2.7%	19.0%	39.2%	20.1%	47.2%	24.0%	47.2%	25.0%	44.1%	25.4%	43.7%	26.2%	43.7%	25.4%	43.7%
	3	6.4%			0.0%	4.3%	2.0%	13.0%	2.8%	15.9%	1.6%	15.5%	2.4%	15.4%	2.8%	14.3%	2.8%	12.8%
	4	0.5%					0.0%	3.9%	0.4%	6.6%	1.2%	9.1%	1.2%	10.0%	1.2%	10.4%	1.6%	10.8%
	5	0.5%					0.0%	0.4%	0.0%	3.5%	0.0%	4.8%	0.4%	7.5%	0.4%	7.5%	1.2%	9.4%
	6	0.0%									0.0%	2.4%	0.0%	3.2%	0.0%	4.8%	0.0%	6.0%
	7	0.0%											0.0%	1.6%	0.0%	2.4%	0.0%	2.8%
	8	0.0%											0.0%	0.4%	0.0%	0.8%	0.0%	0.8%
	9	0.0%													0.0%	0.4%	0.0%	0.4%
Pool	50	Observed	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Clonal Counts	1	71.8%	96.8%	100.0%	59.1%	80.6%	46.8%	72.4%	41.2%	64.8%	37.6%	62.0%	36.8%	57.6%	35.2%	56.8%	34.4%	55.6%
	2	20.9%	0.0%	3.2%	16.7%	39.7%	21.2%	44.8%	24.4%	44.4%	25.2%	44.8%	25.6%	44.8%	26.0%	44.0%	27.6%	43.6%
	3	6.4%			0.0%	4.3%	2.4%	13.2%	2.4%	15.2%	3.1%	15.6%	3.2%	14.8%	3.2%	14.0%	3.2%	14.0%
	4	0.5%					0.0%	4.4%	0.0%	7.6%	0.8%	10.8%	1.2%	10.4%	1.6%	11.2%	2.0%	10.4%
	5	0.5%							0.0%	3.5%	0.0%	5.2%	0.0%	6.8%	0.4%	8.4%	0.4%	10.0%
	6	0.0%									0.0%	2.4%	0.0%	3.6%	0.0%	4.4%	0.0%	5.6%
	7	0.0%											0.0%	1.2%	0.0%	2.4%	0.0%	2.8%
	8	0.0%													0.0%	0.8%	0.0%	0.8%
	9	0.0%																

Table S1 compares the observed ratio of germ lines with those predicted by our model. The upper half of the table shows model results when germ line precursors are drawn at random from 20 adjacent nuclei; the lower half shows results when precursors are drawn from a pool 50. The larger pool size simulates a more random selection of nuclei from the syncytium. Row headings indicate clonal count of one through nine. The observed data appear in the third column. Column headings indicate the number of progenitor nuclei modeled, with the minimum and maximum model results obtained for each out of a thousand independent simulations for each pair of variable. Model predictions with a minimum and maximum of zero are left empty. Cells highlighted in green indicate when the observed values fall between the minimum and maximum model predictions, including those cases when the observed results were zero.

Global Considerations

Extent and Nature of Alkylation: Aaron and Lee (1978) isolated sperm from flies exposed to radio-labeled EMS and measured label incorporation. They estimated that 1.4% of the nucleotides in the sample were alkylated. Since the majority of alkylations involve purines, it follows that nearly 1.4% of the nucleotide pairs were labeled. Table S2 tabulates data from Singer and Grunberger (1983) on the various adducts formed by *in vitro* alkylation of DNA; they noted that similar results are found for DNA alkylated *in vivo*. Sequencing of DNA from flies carrying mutagenized chromosomes (Bentley *et al.* 2000; Cooper *et al.* 2008; Blumenstiel *et al.* 2009) provided estimates of 2–5 sequence changes per million base pairs. Results from Aaron and Lee (1978) imply 14,000 alkylations per million base pairs:

Table S2 Alkylation products of *in vitro* exposure of DNA to EMS (Singer and Grunberger 1983).

Alkylation site	% of alkylations
Adenine	
N-1	1.7
N-3	4.9
N-7	1.1
Σ	7.7
Guanine	
N-3	0.9
O-6	2
N-7	65
Σ	67.9
Cytosine	
N-3	5
Diester	10
Σ	15
$\Sigma\Sigma$	90.6

Transcription data from five-day-old *Drosophila* males contained information regarding more than 15,000 transcripts of which more than 14,000 (14,666) appeared to be protein-encoding genes. The distribution of sizes of genes encoding these transcripts was skewed with half of them under 2kb in length and the remainder distributed in diminishing numbers over 395 kb for a total of approximately 91 million base pairs.

Genomic Effects – Many Alterations, Few Mutations: It has been estimated that there are approximately 3,600 vital genes (among 14,666) in the *Drosophila* genome (Lefevre and Watkins 1986; Brizuela *et al.* 1994 Miklos and Rubin 1996). Rounding these numbers to 600 on the *X* and 1500 on each autosome, we estimate our results concern mutation of 3,000 vital genes. Assuming that the vital genes are representative of the other *Drosophila* genes, they comprise one-fifth of the 91-million base pairs in the total genome. At 2 to 5 EMS-induced changes per million base pairs, we can estimate 36 to 90 changes in vital genes per target genome.

Three thousand vital genes comprising approximately 20% of 91 million base pairs results in 18.2 million base pairs, of which about 1.4% (255 thousand) At $(2 \text{ to } 5) \times 10^{-5}$ altered base pairs among 18.2×10^6 base pairs, there will be 2 to 5 alterations among 14,000 alkylated base pairs. Since there are an estimated 254,800 alkylated base pairs in the target genome, these ratios suggest that 36–120 of them will be abnormal (i.e., mutagenic). This number far exceeds the mutation rates observed. The alkylated base pairs could display eight different adducts of unknown individual effects, some of which may not lead to mutations. Some authors postulate the O-6 alkylation of guanine (2% of the adducts) as the culprit (Vogel *et al.* 1985). Alkylated bases may also fall in nontranslated portions of the

gene, since observed gene lengths used in the calculations are much greater than those required to encode a protein, affect redundant codons, or actually result in altered proteins in ways that do not alter phenotype.

Reexamining Previous Studies in Light of the Model Presented Here

Experiments Involving Specific Locus Mutations

In previous experiments, assessment of the germ line has rarely been followed further than showing whether or not the germ line contains the mutation (Table S3). Lee *et al.* (1970) examined mutations of *yellow*. We discuss this here because *yellow* provides greater anatomical coverage than other loci and can therefore be more accurately scored for somatic mosaics. They found that of 114 *yellow* mutations among 54,243 F₁ females, 16 were complete and presumably premitotic in origin (14% compared with our estimate of 12% for complete lethal mutations). The germ lines of 55 of the 98 mosaic females were examined by progeny testing. Among 55 F₁ females somatically mosaic for *yellow*, the ovaries of 17 were wild type, 33 were *yellow*, and 4 were mosaic. The line separating the two domains of the mosaic bisected the presumptive germline progenitors in fewer than 10% of the mosaic embryos. These numbers suggest that 14% of the *y* mutations were premitotic and that the remaining 86% were mitotic mutations. These results cannot be easily compared to the results predicted by our model; the mutation rate for a single locus is low but the Lee *et al.* (1970) data regard only females in which a mutation has certainly occurred. Their hypothesis, based on the numbers of imaginal discs affected, was that *yellow* mutations occur repeatedly during syncytial mitoses with a probability of 0.54

misreplications per replication of y^+ , compared to our estimate of 0.68 per replication of 3,000 vital genes in males.

Table S3 Specific-locus data.

Reference	Locus	Mutagen	Maternal <i>mus</i> constitution	Complete	Somatic mosaic	Total	% Mutant
Auerbach (1946 a and b)	<i>M</i>	mustard	+	21	17	4,837	0.79
	<i>M</i>	X ray	+	20	1	4,161	0.50
	<i>w sn</i>	mustard	+	12	6	15,300	0.12
	<i>w sn</i>	X ray	+	6	0	3,600	0.17
Carlson and Oster (1962)	<i>dp</i>	ICR100 ^a	+	23	163	21,102	0.88
	<i>dp</i>	X ray	+	137	65	?	
Jenkins (1967a and b)	<i>dp</i>	EMS	+	10	36	7,131	0.65
	<i>dp</i>	EMS	+	34	120	22,388	0.69
Lee <i>et al.</i> (1970)	<i>y</i>	EMS	+	16	98	50,243	0.23
	<i>w</i>	EMS	+	111	106	50,243	0.43
Ondrej (1971)	<i>dp</i>	ENU	+	12	38	14,095	0.35
				34	63	19,345	0.50
				29	52	12,677	0.64
Pastink <i>et al.</i> (1991)	<i>v</i>	EMS	+	21	^b	155,196	0.013531
					13 ^c	25,525	0.0509
	<i>v</i>	EMS	<i>mus201^{D1}</i>	16	^b	94,425	0.0169
					6 ^c	15,624	0.0384

^a 2-methoxy-6-chloro-9-(3[ethyl-2-chloroethyl] aminopropylaminoacridine dihydrochloride

^b mosaics not detectable in F₁ owing to non autonomy of *v*

^c mosaics identified in F₂

Experiments Involving Recessive Lethal Mutations on the X Chromosome

Recessive lethal tests score simultaneously for mutations in large numbers of vital genes (e.g., approximately 600 vital genes on the X). Only germ-cell constitutions are testable in these crosses; somatic constitutions cannot be determined because the mutagenized chromosomes are recovered and maintained in balanced heterozygotes. Virtually all recessive-lethal screens have dealt with the X chromosome, using the standard *Basc* method. Sex-linked-recessive-lethal data are complex. Mutations designated as complete lethals are recognized by the absence of sons among the offspring of daughters of mutagen-treated males. This class comprises prefertilization mutations and products of monoclonal mutant germ lines, as well as those classes derived from polyclonal gonads of the types scored as double and triple mosaics (i.e., LL and LLL) in our experiment; lethal mutations of the latter source will actually be a mixture of two or three different lethal mutations. The mutagenized X chromosome is recovered in F₁ heterozygous females, and whole-gonad mutations are detected by the absence of sons carrying the mutagenized X in the F₂ generation. Gonadal mosaics for lethal mutations are hidden among the F₁ females not heterozygous for a complete lethal. They could be gonadal mosaics, whose effect on the F₂ could be a skewed sex ratio in favor of daughters owing to the fact that half of the sons receive a lethal-bearing X chromosome; this datum is not ordinarily recorded. Instead F₁ females are again backcrossed to males and the F₂ daughters of gonadal mosaics will comprise a mixture of lethal and nonlethal heterozygotes (Table S4). A number of daughters must be tested individually for the presence of lethality in the F₃; the daughters of gonadal mosaics segregate for lethal mutations. This procedure can be extended for another generation to reveal lethal mutations in the F₄ (Mathew 1964). A shortcoming of

sex-linked-lethal mutation data is that they do not ordinarily admit to complementation analysis for determination of allelism of the mutations recovered. (Note: infrequent lethal mutations located in the region at the base of the X chromosome that is covered by the pseudo-autosomal region *Dp(1;Y)mal⁺* can and have been complementation tested by Lifschytz and Falk (1969).)

In an early study, Auerbach (1946a and b) selected 11 gonadal mosaic daughters of mustard-gas-treated males. From these females she progeny-tested an average of 18 F2 females each. All F1 females tested produced a mixture of **L** and **P** daughters (139:47). This preponderance of lethals probably biased the selection of these F1 females as putative gonadal mosaics. Surprisingly, four of the F1 females also produced one or two gonadal mosaic daughters. Auerbach provided evidence that the mosaic lethal daughters of mosaic lethal mothers were mosaic for the same lethal gene, on the basis of similarity of lethal phenotype and map position in one case and a garnet mutation in another. It appeared that at least six out of 11 gonadal mosaic females tested were carrying replicating instabilities that stabilized as lethal mutations in some of their daughters. The observation that gonadal mosaic females can produce gonadal mosaic daughters suggests that there is no reason that a specific lethal-bearing nuclear type cannot continue to be propagated in future generations.

Table S4 Sex-linked recessive lethal mutations.

Reference	Mutagen	Maternal <i>mus</i> constitution	F2 Complete mutations			F3 Mosaics		
			Non	Gonadal complete	% Complete	Non	Gonadal mosaic	% Mosaic
Carlson and Oster (1962)	ICR 100	+	295	11	3.6	101	16	13.7
	X ray	+	351	54		285	3	
Carlson and Southin (1963)	ICR 100	+	821	97	9.2	563	91	13.9
	ICR 100	+	688	69	9.1	346	67	16.2
Mathew (1964)	CEMS	+	762	15	1.9	54	2	3.6
Ondrej (1971)	ENU	+	513	29	5.3	550	228	29.3
Sadiq and Mathew (1977)	MNNG	+	950	68	6.7	158	7	4.2
	MNNG	+	890	62	6.5	125	3	2.3
Shvartsman and Romashkina (1995)	Ethylenimine	+	585	6	1			12.4 ^c
		<i>mus201</i> ^{G1}	435	46	9.6			49.2 ^c
	Ethylenimine	+	595	23	3.7			
		<i>mus201</i> ^{G1}	640	178	21.8			
	MMS	+	801	21	2.6			
		<i>mus201</i> ^{G1}	1080	93	7.9			

^a Chloroethylmethane Sulfonate^b N-Methyl-N'-Nitro-Nitrosoguanidine^c raw numbers not given (percentage only)

Experiment Involving Autosomal Recessive Lethal Mutations

Autosomal recessive lethals exhibit the same complexity of origin as sex-linked recessive lethals except that double and triple mosaics give rise to normal rather than lethal offspring. They differ from sex-linked recessive lethals in allowing for complementation testing of the mutations. Epler (1966) performed the first screen for autosomal lethal mutations (Table S5). He screened both chromosomes 2 and 3 in the same experiment. He assessed the cellular contents of the germ lines of the sons of EMS-treated males by

establishing 10 lines from each; among these he encountered single mosaics containing some lethal-bearing and some nonlethal lines as well as double mosaics carrying complementing-lethal lines. This first demonstration of multiple mosaicism established the value of complementation testing, and the first suggestion that multiple mosaics likely result from independent misreplications in the generation of multiple clones in biclonal and triclinal germ lines.

The results of the Epler experiment are consistent with our model assuming three nuclei selected as germline progenitors and a mutation rate of approximately 25%.

Table S5 EMS-induced autosomal recessive lethal mutations (Epler 1966).			
Chromosome	Non (P)	Gonadal complete (L)	Gonadal mosaic (M)
2	35	9	7
3	21	11	7
Σ	56	20	14
Σ%	62%	22%	16%

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