File S2

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
∑	**7.7**
Guanine	
N-3	0.9
O-6	2
N-7	65
∑	**67.9**
Cytosine	
N-3	5
Diester	10
∑	**15**
∑∑	**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 to 5)×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 F_1 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 *mus* constitution	Complete	Somatic mosaic	Total	% Mutant
Auerbach (1946 a and b)	*M*	mustard	+	21	17	4,837	0.79
	M	X ray	+	20	1	4,161	0.50
	w sn	mustard	+	12	6	15,300	0.12
	w sn	X ray	+	6	0	3,600	0.17
Carlson and Oster (1962)	*dp*	ICR100[a]	+	23	163	21,102	0.88
	dp	X ray	+	137	65	?	
Jenkins (1967a and b)	*dp*	EMS	+	10	36	7,131	0.65
	dp	EMS	+	34	120	22,388	0.69
Lee *et al.* (1970)	*y*	EMS	+	16	98	50,243	0.23
	w	EMS	+	111	106	50,243	0.43
Ondrej (1971)	*dp*	ENU	+	12	38	14,095	0.35
				34	63	19,345	0.50
				29	52	12,677	0.64
Pastink *et al*. (1991)	*v*	EMS	+	21	[b]	155,196	0.013531
					13[c]	25,525	0.0509
	v	EMS	*mus201*^{D1}	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 F_1 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 F_1 heterozygous females, and whole-gonad mutations are detected by the absence of sons carrying the mutagenized *X* in the F_2 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 F_2 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 *mus* constitution	F2 Complete mutations Non	Gonadal complete	% Complete	F3 Mosaics 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]
		mus201^{G1}	435	46	9.6			49.2[c]
	Ethylenimine	+	595	23	3.7			
		mus201^{G1}	640	178	21.8			
	MMS	+	801	21	2.6			
		mus201^{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 triclonal 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%**

Literature Cited

Bentley, A., B. MacLennan, J. Calvo, and C. R. Dearolf, 200O Targeted recovery of mutations in *Drosophila*. Genetics **156**: 1169–1173.

Blumenstiel, J. P., A. C. Noll, J. A. Griffiths, A. G. Perera, K. N. Walton, W. D. Gilliland, R. S. Hawley, and K. Staehling-Hampton, 2009 Identification of EMS-induced mutations in *Drosophila melanogaster* by whole-genome sequencing. Genetics **182**: 661–667.

Brizuela, B. J., L. Elfring, J. Ballard, J.W. Tamkun, J.A. Kennison, 1994 Genetic analysis of the *brahma* gene of *Drosophila melanogaster* and polytene chromosome subdivisions. 72AB. Genetics **137**: 803-813.

Carlson, E.A. and I. I. Oster, 1962 Comparative mutagenesis of the dumpy locus in *Drosophila melanogaster*. II. Mutational mosaicism induced without apparent breakage by a monofunctional alkylating agent. Genetics **47**: 561–576.

Carlson E.A, Southin, J. L., 1963 Chemically induced somatic and gonadal mosaicism in *Drosophila* I sex-linked lethals. Genetics Vol **48**: 663-675.

Cooper, J. L., E. A. Greene, B. J. Till, C. A. Codomo, B. T. Wakimoto and S. Henikoff, 2008 Retention of induced mutations in a *Drosophila* reverse-genetic resource. Genetics **180**: 661–667.

Jenkins, J. B., 1967a The induction of mosaic and complete dumpy mutants in *Drosophila melanogaster* with ethyl methanesulfonate. Mutation Res. **4**: 90–92.

Jenkins, J. B., 1967b Mutagenesis at a complex locus in *Drosophila* with a monofunctional alkylating agent, ethyl methanesulfonate. Genetics **57**: 783–793.

Lefevre, G., Watkins, W.S. 1986. The question of the total gene number in *Drosophila melanogaster*. Genetics **113**: 869--895

Lifschytz, E., and R. Falk, 1969 Fine structure analysis of a chromosome segment in *Drosophila melanogaster* analysis of ethane methyl sulphonate-induced lethals. Mutation Res. **8**: 147–155.

Miklos, G. L. and Rubin, G. M., 1996 The role of the genome project in determining gene function: insights from model organisms. Cell **86**, 521-529.

Ondrej, M., 1971 The proportion of complete mutations, mosaics and instabilities induced by ethyl nitrosourea in *Drosophila melanogaster*. Mutation Res. **12**: 159–169.

Pastink, A., E. Heemskirk, M. J. M. Nivard, C. J. van Vliet, and E. S. Vogel, 1991 Mutational specificity of ethyl methanesulfonate in excision-repair-proficient and –deficient strains of *Drosophila melanogaster*. Mol. Gen. Genet. **229**: 213–218.

Sadiq, M. F., and C. Mathew, 1977 Production of mosaic lethals in different germ cell stages of Drosophila by N-methyl-N'-nitro-N-nitrosoguanidine. Mutation Res. **45**: 31–39.

Shvartsman, P. Ya., and T. B. Romashkina, 1995 Maternal effect of the mutagen-sensitive mutation mus(2)201^{G1} on mutagenesis induced by etylenimine and methyl methanesulfonate in the genome of sperms in *Drosophila melanogaster*. Genetika **31**: 205–208.

Singer, B., and D. Grunberger, 1983 Molecular biology of mutagens and carcinogens. New York, Plenum Press. 347 pp.

Vogel, E. W., R. L. Dusenbery, and P. D. Smith, 1985 The relationship between reaction kinetics and mutagenic action of monofunctional alkylating agents in higher eukaryotic systems IV. The effects of the excision-defective *mei-9^{L1}and mei201^{D1}* mutants on alkylation-induced genetic damage in *Drosophila*. Mutation Res. **149**: 193–207.