Molecular Analysis of Mutations Induced in the *vermilion* Gene of *Drosophila melanogaster* by Methyl Methanesulfonate

Madeleine J. M. Nivard, Albert Pastink and Ekkehart W. Vogel

MGC-Department of Radiation Genetics and Chemical Mutagenesis, State University of Leiden, Sylvius Laboratories, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands

Manuscript received November 18, 1991
Accepted for publication March 28, 1992

ABSTRACT

The nature of DNA sequence changes induced by methyl methanesulfonate (MMS) at the *vermilion* locus of *Drosophila melanogaster* was determined after exposure of postmeiotic male germ cell stages. MMS is a carcinogen with strong preference for base nitrogen alkylation ($s = 0.86$). The spectrum of 40 intralocus mutations was dominated by AT → GC transitions (23%), AT → TA transversions (54%) and deletions (14%). The small deletions were preferentially found among mutants isolated in the F_1 (8/18), whereas the AT → GC transitions exclusively occurred in the F_2 (6/22). The MMS-induced transversions and deletions are presumably caused by *N*-methyl DNA adducts, which may release apurinic intermediates, known to be a time-related process. Furthermore, MMS produces multilocus deletions, *i.e.*, at least 30% of the F_1 mutants analyzed were of this type. A comparison of the mutational spectra of MMS with that produced by ethylnitrosourea (ENU), also in the *vermilion* locus of Drosophila, reveals major differences: predominantly transition mutations (61% GC → AT and 18% AT → GC) were found in both the F_1 and F_2 spectrum induced by ENU. It is concluded that the mutational spectrum of MMS is dominated by nitrogen DNA adducts, whereas with ENU DNA sequence changes mainly arose from modified oxygen in DNA.

THE general relationship between nucleophilic selectivity of monofunctional alkylating agents and induced genetic effects in *Drosophila melanogaster* has been a subject of intense investigation for more than 10 years (VOGEL and NATARAJAN 1979a,b; VOGEL *et al.* 1986). These studies suggested a positive correlation between the relative ability of an agent for *N*-alkylation in DNA, expressed by the O^6-/*N*-7-alkylguanine ratio (LAWLEY 1974; SINGER and GRUNBERGER 1983), and its efficiency for structural chromosome aberrations, such as translocations, deletions or chromosome loss. Point mutations by these agents were thought to arise from apurinic sites (AP-sites) (LOEB and PRESTON 1986). Chemicals showing preference for *O*-alkylation in DNA caused high frequencies of mutations, whereas chromosomal aberrations were relatively rare (VOGEL 1986). These results were in line with similar studies conducted earlier on plant systems (OSTERMAN-GOLKAR, EHRENBERG and WACHTMEISTER 1970).

The molecular techniques developed in more recent years to analyze genetic alterations at the DNA level provide more exact information on agent-specific DNA modifications. A number of such studies have dealt with sequence analysis of mutations induced by small alkylating agents in both prokaryotic and eukaryotic systems (DUBRIDGE *et al.* 1987; RICHARDSON *et al.* 1987; BURNS, GORDON and GLICKMAN 1988; ECKERT *et al.* 1988; HORSFALL *et al.* 1989). Data from sequence analysis of mutants induced by small alkylating agents in Drosophila have previously been reported by BATZER *et al.* (1988), GRAY *et al.* (1991) and our group (PASTINK *et al.* 1989) for ethyl nitrosourea (ENU), an agent with low nucleophilic selectivity ($s = 0.26$), and for ethyl methanesulfonate (EMS), an agent of moderate nucleophilic selectivity ($s = 0.67$) (PASTINK *et al.* 1991). However, most of these studies were performed using alkylating agents with low nucleophilic selectivity, *i.e.*, with low Swain-Scott *s* values.

Against this background, the primary objective of this study was to elucidate for an agent of high *s* value some of the sequential events taking place between DNA adduct formation and manifestation of genetic damage, using the *vermilion* gene of *D. melanogaster* for mutation induction and molecular analysis. Methyl methanesulfonate (MMS, $s = 0.86$), in contrast to ENU, is very poor in alkylating oxygen in DNA, whereas more than 99% of all DNA lesions are on nitrogen (SINGER and GRUNBERGER 1983; BERANEK 1990). It has been postulated that products such as 7-methylguanine and 3-methyladenine, if unrepaired or misrepaired, could be potentially mutagenic lesions causing both mutations and chromosomal aberrations (OSTERMAN-GOLKAR, EHRENBERG and WACHTMEISTER 1970; LAWLEY 1974; VOGEL and NATARAJAN 1982). Depurination of these *N*-methyl DNA lesions will give apurinic sites (SINGER and GRUNBERGER 1983). If these led to mutations, specific base-pair

changes would be expected, *i.e.*, adenine opposite guanine or adenine, respectively (SCHAAPER, KUNKEL and LOEB 1983; KUNKEL 1984). Another aspect of DNA damage processing after *N*-alkylation concerns the differential persistence of the main *N*-methyl DNA adducts. It is therefore important to determine whether the relative frequency of AP site-mediated mutations changes with time. There are two aspects with respect to the parameter time which can be studied in *D. melanogaster*. First, the time interval between DNA adduct formation in the male germ cell and the first DNA replication, which takes place after fertilization, can be altered. Second, a distinction can be made between mutants which are caused by immediate mutation fixation, termed F_1 mutants, and those where the mutation is fixed after a few rounds of DNA replication, named mosaics or F_2 mutants.

MATERIALS AND METHODS

Induction and isolation of *vermilion* mutants: For the induction of *vermilion* mutants 30 *bw* males were placed in plastic vials containing 8 layers of glass microfiber paper (Whatmann GF/A) soaked with 0.9 ml of a MMS (Merck-Schuchardt) solution in 33 mM phosphate buffer (pH 6.8; 16.5 mM Na_2HPO_4, 16.5 mM KH_2PO_4) containing 5% sucrose. The concentration of MMS varied from 0.25 mM to 3.0 mM. After 24-h exposure the males were mated in bottles to *In(1) sc^{S1L} sc^{8R} In(1)dl-49, y sc^{S1} sc^{8} v; bw* virgins (*Inscy, v; bw*) (brood 1A) (LINDSLEY and GRELL 1967), at a sex ratio of 1:1. Fractionation into broods was as follows: males were remated to virgin females at 48-h intervals, to yield 1A and 2A. Females from broods "A" were transferred to new bottles for 48–72 h, to yield B and C broods, respectively.

The F_1 female progeny of the various broods was screened for the occurrence of *vermilion* mutations. Because *vermilion* is nonautonomous, F_1 individuals carrying both mutated and nonmutated tissue do not express the mutant phenotype. However, if at least part of the germ cells carries the new mutation, these "delayed" mutants can be picked up in the F_2 generation. Therefore, phenotypically nonmutated F_1 females were crossed with their brothers either in mass cultures in bottles or at a 1:1 ratio in vials, in order to determine in the same experiment the frequency of sex-linked recessive lethal mutations. Of the F_2 generation both males and females were screened for occurrence of newly induced *vermilion* mutations.

Mutant F_1 or F_2 females, for two reasons were crossed to wild-type Berlin K males. First, to make the flies heterozygous for the *brown* marker and thereby express the vermilion phenotype. Second, to delete by crossing over recessive lethals which some of the *vermilion* mutants carried on their *X* chromosome. A crossing over event between *vermilion* and a lethal mutation somewhere on the *X* chromosome will produce a "male viable" *vermilion* mutant, and in this way a new strain homozygous for the mutation could be built up. In cases where the *vermilion* mutation itself was associated with a recessive lethal mutation, or when the lethal and *vermilion* mutation were two independent events but situated closely together on the *X* chromosome, a heterozygous strain was constructed by keeping the chromosome carrying the lethal and the *vermilion* mutations opposite a chromosome which in its *vermilion* locus had an insert of around 1500 bp (v^{Amh}).

Molecular analysis: The *vermilion* gene is located on the *X* chromosome, has a size of 2 kb and codes for a mRNA transcript of 1.4 kb (SEARLES *et al.* 1990). For sequencing the coding region of the *vermilion* gene, the locus was cloned in a vector containing an M13 origin of replication. By this method single-stranded DNA (ssDNA) of the *vermilion* gene could be isolated, which is needed for sequence analysis utilizing the dideoxy methodology. Two different cloning procedures were used: (i) a method based on the recombinational screening technique developed by SEED (1983) and (ii) a polymerase chain reaction (PCR) procedure described by PASTINK *et al.* (1989, 1991). With a few modifications, the latter procedure enabled an analysis of DNA from single flies that were sterile. DNA from a sterile mutant was amplified, digested, ligated in the vector M13 mp11 and transfected as previously described (PASTINK *et al.* 1991). However, the amplified DNA of sterile flies can contain two different PCR products: (i) amplified DNA of the *vermilion* gene of the untreated (maternal) *Inscy, v* chromosome and (ii) amplified DNA of the *vermilion* gene containing the newly induced mutation. The *vermilion* gene of the *Inscy* chromosome contains several base pair alterations compared to the *vermilion* gene located on the (paternal) *X* chromosome of the mutagenized *bw* males. These changes include nucleotides 451 (AT → GC), 610 (AT → TA), 1030 (AT → TA), 1105 (GC → AT), 1114 (AT → GC) and a 15-bp deletion between positions 652 and 669. The presence of this 15-bp deletion has been used in the analysis of new *vermilion* mutants that were sterile. After transfection of the M13 mp11 vector ligated with the PCR product into PC2495 cells, two types of phages can be expected, *i.e.*, one carrying DNA from the *Inscy* chromosome and the other carrying DNA from a copy of the treated *X* chromosome. To distinguish these two types of phages, filter-hybridization was performed using two different probes. From every plate two filter replicas were made: one was hybridized with the *vermilion* fragment pv8.3 (PASTINK *et al.* 1989) and the other with primer pLMN (5′ GGT TTT CAG TCG CTG CAG TTC CG 3′) covering the 15-bp fragment (underlined), which is deleted in the *vermilion* locus of the *Inscy* chromosome. Phages hybridizing with both pv8.3 and pLMN carried *vermilion* DNA of the paternal *X* chromosome and were used for ssDNA isolation and sequence analysis.

In cases where mutants were male lethal, a heterozygous strain was built by keeping the *vermilion* mutation opposite a v^{Amh} chromosome. Because of the presence of a 1500-bp insert in the v^{Amh} locus, the *vermilion* allele of the treated (paternal) *X* chromosome was cloned selectively in M13 mp11 after amplification.

Cytological analysis: Polytene chromosomes of the salivary glands were studied from those mutants giving neither a PCR product after amplification nor hybridization with *vermilion* probes, after Southern blot analysis (PASTINK *et al.* 1989). The chromosome containing the *vermilion* mutation was placed opposite a chromosome carrying the markers y^2, *sn, lz, ras, v, m* (LINDSLEY and GRELL 1967). The salivary glands were isolated from third instar larvae of these heterozygous females. The isolation procedure of polytene chromosomes has been described by PARDUE (1986).

RESULTS

Genetic analysis: Treatment of postmeiotic stages of *D. melanogaster* males with 0.25–0.35 mM MMS induced a 15-fold increase (2.1%) in the sex-linked recessive lethal (SLRL) mutation frequency over the spontaneous background (0.14%). A higher exposure

TABLE 1

Induction of *vermilion* mutants following treatment of postmeiotic male germ cells with MMS for 24 hr

Concentration (mM)	Offspring scored for *vermilion* in F_1 or F_2	Transmissible *vermilion* mutants			Sterile F_1 mutants[a]	*vermilion* mutation frequency ($\times 10^{-4}$)	SLRL frequency (per locus $\times$ 10^{-4})[b]
		Male viable	Male lethal	Total			
Control	F_1 105,274	0	0	0	0	<0.095	0.02
	F_2 11,907[c]	0	0	0		<0.8	
1.5–3	F_1 211,832	12	7[d]	19	10	0.9	1.4
	F_2 16,982[c]	2	1	3		1.8	
0.5	F_1 232,416	5	4[e]	9	4	0.4	0.6
	F_2 10,238[c]	5	0	5		4.9	
0.25–0.35	F_1 77,115	2	0	2	0	0.3	0.3
	F_2 11,899[c]	3	0	3		2.5	
Total: 0.25–3	F_1 521,363	19	11	30	14		
	F_2 39,119[c]	10	1	11			

[a] Phenotypically F_1 mutants which were sterile are not included for the calculation of the *vermilion* mutation frequency.
[b] SLRL mutation frequencies are divided by 700 to obtain the average mutation frequency per locus.
[c] Steriles are not included.
[d] Two of seven "male-lethal mutants" had very low fertility, for one mutant the lethal mutation could be removed by crossing over.
[e] From two of the four "male-lethal mutants" the lethal mutation could be separated from the induced *vermilion* mutation by a crossing over event.

The overall percentages of sterile F_1 females were: control 4.8%, 1.5–3 mM MMS 10%, 0.5 mM MMS 7.3% and 0.25–0.35 mM MMS 5.2%.

dose of 1.5–3 mM MMS produced a 70-fold increase (9.8%). The frequency with which *vermilion* mutations occur spontaneously has not yet been determined. SCHALET (1986) calculated the average spontaneous mutation rate for 13 vital visible loci in *D. melanogaster* to be between 2.3 and 3.5×10^{-6}. In the same study, a value of $1.8–3.8 \times 10^{-6}$ was estimated for the average recessive lethal rate per locus. Therefore, in Table 1, the frequency of SLRL has been divided by 700, providing the average mutation frequency per locus. This calculation is based on the estimate that there are 700 loci on the *X* chromosome which can give rise to an SLRL (ABRAHAMSON *et al.* 1980). Based on this figure the spontaneous recessive lethal mutation frequency per locus was 2×10^{-6}, which is in agreement with the data of SCHALET. For the induction experiments with MMS, for both recessive lethals and F_1 *vermilion* mutants, the per locus frequencies are of the same order, at all MMS doses (Table 1). For the dose range tested, frequencies of *vermilion* mutations were 2–20-fold higher in the F_2 compared to the F_1.

As can be seen from Table 1, F_1 and F_2 flies showing a *vermilion* phenotype fall into three classes: (i) male viable, *i.e.*, 29 mutants with a transmissible *vermilion* mutation but without any recessive lethal mutation on the *X* chromosome; (ii) male lethal, *i.e.*, 12 mutants carrying on the treated *X* chromosome a recessive lethal in addition to a *vermilion* mutation. For some of these mutants, the two mutations were located at distinct sites on the chromosome and could be separated from each other by crossing over experiments. In this way, strains homozygous for the *vermilion* mutation could be built up for three male lethal mutants (218, 250 and 251; Table 2); and (iii) sterile, *i.e.*, "phenotypically mutated" F_1 females which were sterile. This class consisted of 14 mutants, but they are not included in the frequency calculations because the mutations could not be verified at the genetic level (Table 1).

Molecular analysis: In the first part of molecular analysis, 40 male viable mutants were characterized (Tables 2 and 3). Of these 40 mutants, 25 were from the "male viable class" specified in Table 1, three mutants originally had been "male lethal" and additional 12 "male viable" mutants came from F_2-mass-culture experiments (see MATERIAL AND METHODS). Eighteen mutants were isolated from the F_1 and 22 from the F_2 generation. Only one mutant (236) showed an altered restriction pattern with Southern blot analysis, namely a 150-bp deletion located at the start of the coding sequence. The other 39 mutants were caused by DNA alterations including all types of base substitutions, deletions smaller than approximately 50 bp, and one mutant (242), classified as deletion/insertion, had a change in four or five nucleotides adjacent to each other. All mutations were located inside of an exon or covered at least part of an exon. The point mutations always gave rise to an amino acid change or to a nonsense codon. A clear difference in the relative distribution of the various DNA sequence alterations was found between F_1 and F_2 mutants. Among the F_1 mutants 39% were deletions (210, 217, 226, 208, 251, 281, 252). More than

TABLE 2

Mutations induced by MMS in the *vermilion* gene of *D. melanogaster*

Mutants	Concn. (mM)	Brood	Position	Change	Amino acid	Target sequence 5′–3′
MMS F_1 mutants						
207	2	1A	578	GC → CG	Gln → Glu	TGGAC C AAGTG
227	2	1A	1149	AT → TA	Asp → Val	CATCG A CTCGT
245	2	1A	678	AT → TA	Ile → Asn	TTTGA T CGAGA
210	2	1B	1021–1036	16-bp deletion		No tandem repeats
220	2	1B	390	GC– → AT	Trp → UAG	GCTTT G GTTCA
217	2	1C	247–262[a]	14-bp deletion		*–CC–––CC–* → *–CC–*
218	2	1C	704	GC → TA	Gln → Tyr	TGACA G AGCAG
226	2	1C	639–646[a]	6-bp deletion		*–CA–––CA–* → *–CA–*
246	2	1C	1270	AT → TA	His → Leu	CAATC A CGTGA
257	0.5	1C	492	GC → CG	Arg → Pro	GAACC G AGTGG
208	2	2A	300–307[a]	3-bp deletion		–CATCATC– → –CATC–
242	2	2A	1435–1439	5-bp del/insert		TGATC → AACA*N*[b]
250	0.5	2A	1265	GC → TA	Tyr → UAA	GCAGA C AATCA
251	0.5	2A	377–386[a]	8-bp deletion		*–GC–––GC–* → *–GC–*
223	2	2B	836	GC → TA	Gly → UGA	CGCCC G GACTG
281	0.35	2B	1496	−1 frame shift		GCGA (GG) CGAT
291	0.35	2B	404	AT → TA	Ile → Phe	AGATC A TCTTT
252	0.5	2C	665–670[a]	5-bp deletion		–C–––C– → –C–
MMS F_2 mutants						
202	2	1A	240	AT → TA	Leu → Gln	ACTGC T GGATG
214	1.5	1A	996	AT → TA	Ile → Asn	CTCCA T CTTTG
219	2	1A	1002	AT → GC	Asp → Gly	CTTTG A TCCGG
237	2	1A	1327–1352[a]	24-bp deletion		*–CT–––CT–* → *–CT–*
253	0.5	1A	675	AT → CG	Leu → Trp	CCGTT T GATCG
270	0.25	1A	647	AT → GC	Ser → Pro	CCGCA T CTGGT
201	2	1B	996	AT → TA	Ile → Asn	CTCCA T CTTTG
203	2	1B	210	AT → TA	Tyr → Phe	AATCT A TGGAG
233	2	1B	396	AT → GC	Lys → Arg	GTTCA A GCAGA
244	2	1B	1077	AT → TA	Met → Lys	CATCA T GATCA
266	0.5	1B	1171	AT → TA	Arg → Ser	TGGAG A TGTAA
272	0.35	1B	647	AT → GC	Ser → Pro	CCGCA T CTGGT
225	2	1C	503	AT → TA	Ile → Phe	TTCTG A TTCTA
240	2	1C	996	AT → GC	Ile → Thr	CTCCA T CTTTG
249	2	1D	303	AT → GC	Ile → Thr	GTTCA T CATCA
215	2	2A	240	AT → TA	Leu → Gln	ACTGC T GGATG
222	2	2B	1293	AT → TA	Ile → Phe	GCATG A TTGGA
234	2	2B	659	AT → TA	Ser → Thr	TTCAG T CGCTG
238	2	2B	503	AT → TA	Ile → Phe	TTCTG A TTCTA
267	0.5	2B	1442	AT → TA	Tyr → Asn	ATCGG T ACAAG
269	0.25	2B	417	AT → TA	Asp → Val	GTTCG A CTCCA
236	2	2C	0–150	150-bp deletion		

[a] The intragenic deletion consists of a direct repeat adjacent to the breakpoint with one of the repeats deleted.
[b] *N*: unknown nucleotide.

half of them had the deletion between short direct repeats, eliminating one of them and the sequence in between. It is striking that among the 22 F_2 mutants there were only 2 (9%) deletion mutations (236 and 237). The F_2 spectrum was dominated by AT → TA transversions (59% in the F_2 *vs.* 22% in the F_1). There was also a considerable contribution of AT → GC transitions in the F_2 spectrum (27% in the F_2 *vs.* 0 in the F_1). In fact, 20 of the 22 mutants characterized in the F_2 had a base-pair substitution at an AT position.

The results of analyzing 12 sterile and 2 "male lethal" F_1 mutants are summarized in Table 4. For 4 of the 12 steriles the nature of the DNA mutation could be exactly determined: we found 2 AT → TA transversions, 1 GC → TA transversion and 1 AT → GC transition. Another seven sterile mutants seemed to carry a deletion consisting of at least the DNA fragment of primer LMN inside *vermilion* (see MATERIAL AND METHODS), and presumably these deletions also covered the complete gene including the female fertility factor proximal to the *vermilion* locus. With one mutant (M-7) a mutation in the coding region of the gene could not be detected, although 25% of the phages hybridized with primer pLMN (see Table 4). DNA from the two male lethal mutants could not be amplified. Southern blot analysis showed that their DNA did not hybridize with the *vermilion* probes, suggesting that both mutants contained a deletion of

TABLE 3

Classification of 40 MMS-induced *vermilion* mutants

Change	Mutants[a] F1 No.	F1 Frequency (×10⁻⁴)	F2 No.	F2 Frequency (×10⁻⁴)
Transitions				
GC → AT	1	0.023	0	
AT → GC	0		6	0.70
Transversions				
AT → TA	4	0.094	13	1.51
AT → CG	0		1	0.12
GC → TA	3	0.070	0	
GC → CG	2	0.047	0	
Others				
Deletions	7	0.16	2	0.23
Insertions	0		0	
Deletions/insertions[b]	1	0.023	0	
Total no. of mutants	18		22	

[a] Mutation analysis of male lethal and sterile mutants are not included.

[b] Substitution of a few base pairs.

at least the size of the *vermilion* gene. These deletions were not visible in salivary gland analysis of the polytene chromosomes.

Remarkable is the fact that there were four groups, each containing two mutants, carrying at the same position the same base-pair substitution (Table 5). Since all these mutants were induced separately and analyzed in different cycles of isolation, it is unlikely that these duplicates were caused by contaminations. Mutant 240 had a mutation at the same position as two other mutants, but had a different kind of base substitution *i.e.*, AT → GC (240) instead of AT → TA (201, 214).

DISCUSSION

DNA mutation spectra: Data published for bacterial or mammalian cell systems, have shown that the DNA spectra of methylating and ethylating agents of both low and high *s* values were all dominated by GC → AT transition mutations caused by the O^6-alkylguanine adducts (RICHARDSON *et al.* 1987; ECKERT *et al.* 1988; ZIELENSKA, BERANEK and GUTTENPLAN 1988; HORSFALL *et al.* 1990). Illustrative for these observations are the sequence alterations given by dimethylsulfate (DMS) in the *lacI* gene of *Escherichia coli* (ZIELENSKA, HORSFALL and GLICKMAN 1989). Although more than 99% of all DNA modifications by DMS are on nitrogens and oxygen DNA adducts are formed only in minor amounts by this agent, the spectrum was dominated by GC to AT transitions (76%). Similar to the mutational spectra determined for *E. coli* and mammalian cells, GC → AT transition mutations represented the major type of genetic alteration found among *vermilion* mutants after exposure of Drosophila to ethylating agents (Figure 1). With ENU, 61% and 18%, respectively, of the base sequence changes were GC → AT or AT → GC transition mutations (PASTINK *et al.* 1989). The transitions (76%) found with EMS were all of the GC → AT type (PASTINK *et al.* 1991). The origin of these two types of base-pair substitutions can be explained by the direct miscoding properties of respectively O^6-ethylguanine and O^4-ethylthymine (LOECHLER, GREEN and ESSIGMANN 1984; PRESTON, SINGER and LOEB 1986).

The MMS spectrum in *D. melanogaster*, however, showed a pattern which is quite distinct from any mutational spectrum described for bacteria and cells in culture (Table 3). Only 1 out of 40 mutations was a GC → AT transition. Apart from the low amounts of O^6-methylguanine adducts produced by MMS, another reason for the absence of transitions could be the presence in Drosophila of an O^6-methyltransferase protein repairing O^6-methylguanine adducts faster and more efficiently than O^6-ethylguanine adducts. Differences in repair capacity for O^6-alkylguanine adducts have been reported for bacterial and some eukaryotic O^6-alkyltransferases (PEGG, SCICCHITANO and DOLAN 1984; HALL, BRÉSIL and MONTESANO 1985). Further support in favor of a Drosophila O^6-alkyltransferase comes from recent sequence analysis of *vermilion* mutants induced by the methylating agents *N*-methyl-*N*-nitrosourea (MNU), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and dimethylnitrosamine (DMN). Although MNU is a much stronger *O*-alkylating agent than MMS, and in this way comparable with ENU, the MNU spectrum contained only one GC → AT transition out of 26 mutants analyzed. Similar observations were made for MNNG and DMN (M.J.M. NIVARD, A. PASTINK and E. W. VOGEL, unpublished observations). Since the O^6-methylguanine adduct is obviously not a major cause for mutation induction by MMS, other lesions must be responsible. For eukaryotes, there is indirect evidence that *N*-alkylation of DNA, when unrepaired or misrepaired, can give rise to both mutations and structural chromosome aberrations (OSTERMAN-GOLKAR, EHRENBERG and WACHTMEISTER 1970; VOGEL and NATARAJAN 1982). The most frequent sites of attack are the N3 and N7 positions of adenine and guanine. These nitrogen DNA adducts by spontaneous hydrolysis or enzymatic removal of the alkylated base can give apurinic sites (SINGER and GRUNBERGER 1983). The apurinic sites thus formed can subsequently be processed by an excision repair mechanism. When DNA containing an AP site is replicated, a base-pair substitution may be induced. SCHAAPER, KUNKEL and LOEB (1983) and KUNKEL (1984) concluded that in bacterial systems DNA polymerases insert preferentially an adenine opposite a noncoding lesion. If Drosophila polymerases have the same characteristic, the

TABLE 4

Analysis of MMS-induced sterile and male-lethal F_1 *vermilion* mutants

Mutant	Brood (F_1)	Filter hybridization: No. of phage plaques hybridizing with		Mutation	Position	Change
		Fragment 8.3	Primer pLMN			
A. Sterile F_1						
M-3	1A	11	5	AT → TA	392	Phe → Ile
M-4	1B	174	0	Deletion[a]		
M-5	1B	125	0	Deletion[a]		
M-6	2B	37	0	Deletion[a]		
M-7	1B	34	8	No detectable change in coding region		
M-8	2A	21	7	AT → GC	85	Splice site
M-11	2C	10	0	Deletion[a]		
M-12	1B	37	20	GC → TA	693	Gly → Val
M-13	2B	10	0	Deletion[a]		
M-15	2B	20	8	AT → TA	1078	Ile → Phe
M-17	2C	35	0	Deletion[a]		
M-18	2B	45	0	Deletion[a]		

Mutant	Brood (F_1)	PCR product	Southern blot analysis	Cytological analysis
B. Male-lethal F_1				
ML-1	1A	None	Deletion *vermilion* gene	No visible change
ML-2	2C	None	Deletion *vermilion* gene	No visible change

[a] Deletion of the complete *vermilion* gene or of an internal fragment including primer pLMN (see MATERIAL AND METHODS).

TABLE 5

Specific mutations found in more than one *vermilion* mutant

Position	Base pair change	Target sequence 5′–3′	Mutants
240	AT → TA	C-*T*-G	302, 215
503	AT → TA	G-*A*-T	225, 238
647	AT → GC	A-*T*-C	270, 272
996	AT → TA	A-*T*-C	214, 201
996	AT → GC	A-*T*-C	240

AT → TA and GC → TA transversions found in the MMS spectrum are most likely caused by, respectively, the 3-methyladenine and 7-methylguanine adducts giving apurinic sites.

The occurrence of small deletions by MMS was not unexpected in view of the known clastogenic activity of this agent (NATARAJAN *et al.* 1984; VOGEL and NATARAJAN 1979a). Because deletions were not found among *vermilion* mutants produced by ENU, we assumed the responsible adduct(s) for the deletions to be *N*-methylated DNA adducts or their derivatives, such as an apurinic site. It is known that both 3-methyladenine and apurinic sites can block DNA replication (SAGHER and STRAUSS 1983; LARSON *et al.* 1985). In such a case, deletions might be a consequence of incomplete DNA replication. MATSUDA *et al.* (1989) suggested that exposure of paternal germ cells of mice to MMS may cause DNA-strand breaks induced by stress of the chromatin structure after protamine alkylation. Deletions could also be the result of incomplete DNA excision repair. If DNA replication takes place while the excision repair mechanism is still processing, *i.e.*, at the step between the occurrence of an apurinic site and ligation of the repaired strand, a deletion mutation might be formed. With respect to the location of the deletions, five mutants had their deletion between short direct repeats of a length between 2 and 4 bp (Table 2). This type of deletion also occurred among spontaneous or induced deletion mutants at the *white* and *Adh* loci in *D. melanogaster*, the *aprt* locus in hamster cells cultured *in vitro* and at the *LacI* gene in *E. coli* (ALBERTINI

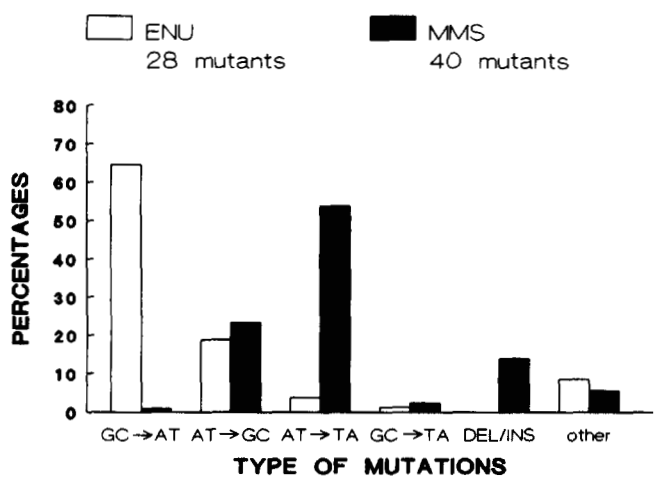

FIGURE 1.—The mutation spectra of transmissible male viable mutations induced by MMS and ENU (PASTINK *et al.* 1989) in the *vermilion* locus. The mutants were isolated both from F_1 and F_2 screens. Percentages of the various mutations were calculated from the mutation frequencies obtained from the F_1 and F_2 analysis (MMS see Table 3).

et al. 1982; BENYAJATI, PLACE and SOFER 1983; BREIMER, NALBANTOGLU and MEUTH 1986; NALBANTOGLU *et al.* 1986; SCHAAPER, DANFORTH and GLICKMAN 1986; LOMONACO, LEE and CHANG 1987; NALBANTOGLU, PHAER and MEUTH 1987; PASTINK *et al.* 1990). These data suggest that repeats play an important function in the formation of deletions. It is further remarkable that 7 of the 9 deletions were isolated in the F_1. It was already mentioned that both 3-alkyladenine and apurinic sites might form blocks for DNA replication and consequently may cause deletions. However, taking into account that the F_2 spectrum was dominated by AT → TA transversions, apurinic sites do not seem to be a good candidate for causing deletions. More relevant for the formation of deletions could be the fact that, after fertilization, around nine nuclear divisions take place relatively fast before the first cell division (FULLILOVE, JACOBSON and TURNER 1978). Consequently, there may be during these first nuclear divisions less time for completing the excision repair pathway and, therefore, a higher chance for induction of a deletion may exist. This further implies that when there is sufficient time for repair, as is probably the case for most of the F_2 mutants, the excision repair mechanism works almost error free. Indeed, it was mentioned before that the AT → TA transversion mutations, which comprise 59% of the F_2 spectrum, are caused by unrepaired apurinic sites and not by repair mistakes.

Mutation spectrum of sterile and male lethal mutants: The work of LEFEVRE (1967, 1969) showed that all multilocus deletions covering the *vermilion* locus were also male lethal, due to a lethal marker just at the left site of this locus. All deletions spanned from *vermilion* to the distal end of the chromosome, which is probably due to the location of a haplo-insufficient female fertility gene on the proximal site of the *vermilion* gene (PASTINK *et al.* 1989). Regarding the MMS experiments, calculations revealed that approximately 50% of the phenotypically screened F_1-mutants were either male lethal or female sterile (Table 1). It is most likely that part of them represented multilocus deletions covering the *vermilion* gene and one or more of the adjacent loci. To verify this hypothesis, 2 "male lethal" and 12 sterile mutants were characterized by an adapted PCR method (see MATERIAL AND METHODS). The result of this analysis suggests that probably more than 50% of the sterile mutants contained a multilocus deletion (Table 4). A comparable percentage of such deletions may be expected among male lethal mutants. Thus, in order not to underestimate the relative frequency of deletions, sterile and male lethal mutants must be included in the analysis. Because only two male lethal mutants were analyzed, the relative proportion of multilocus deletions was estimated on the basis of the total number of sterile and male-lethal mutants induced (Table 1), assuming that approximately 64% (9 out of 14, Table 4) would be large deletions. These calculations resulted in an overall frequency of 32% large deletions among F_1 mutants (Table 6). These observations are in agreement with recent data by CHAUDRY and FOX (1990), showing that 41% of the Chinese hamster *hprt* mutants induced by MMS had lost the entire gene.

TABLE 6

Mutation spectra of MMS-induced F_1 *vermilion* mutants including sterile and male lethal mutants

Type of mutant	No. of mutants		Spectrum of mutations[a]		
	Isolated	Analyzed	Base pair change	Intralocus deletions	Multilocus deletions
Male viable	22	18	12	10	
Male lethal[b]	8	2	3		5
Sterile	14	12	5		9
Overall	44		20 (45%)	10 (23%)	14 (32%)

[a] Data converted to the amount of mutants isolated.
[b] For the molecular data of "male lethal" mutants prognostic values are used based on the spectrum of sterile mutants.

The impact of time on the mutation spectra: From the lack of GC → AT transitions, it is evident that oxygen-methylated DNA adducts do not contribute significantly to the MMS spectrum. The *N*-methylated DNA adducts, especially 3-methyladenine, seem to be much more relevant, *i.e.*, both the AT → TA and GC → TA transversions, and possibly the deletion mutations, may be the result of these *N*-methyl DNA adducts, giving apurinic sites. Formation of AP sites by spontaneous hydrolysis is relatively slow, as the $t_{1/2}$ at 37° and pH 7 is 26 hr for 3-methyladenine and 155 hr for 7-methylguanine (SINGER and GRUNBERGER 1983). Such relatively large time periods are available in Drosophila, as treated male germ cells were stored in females for up to 10 days before fertilization. Interestingly, PARÁDI, VOGEL and SZILÁGYI (1983) found that storage increased the occurrence of deletions after exposure to MMS. By contrast, in prokaryotic and in cellular eukaryotic systems, the time interval between interaction with DNA and mutation fixation is short, *i.e.*, one round of DNA replication. This could be the reason why in these systems mutation induction correlates best with DNA adducts causing direct misincorporation, like oxygen DNA adducts, even in the case of a strong *N*-alkylating agents such as DMS (ZIELENSKA, HORSFALL and GLICKMAN 1989). Thus, the factor time seems an important determinant of mutation induction in both pro- and eukaryotes.

An effect of the parameter time on the process of mutagenesis seems to be the cause of differences between the spectra of F_1 and F_2 mutants. F_1 mutants are caused by direct fixation of the mutation, *i.e.*, before or during the first round of DNA replication;

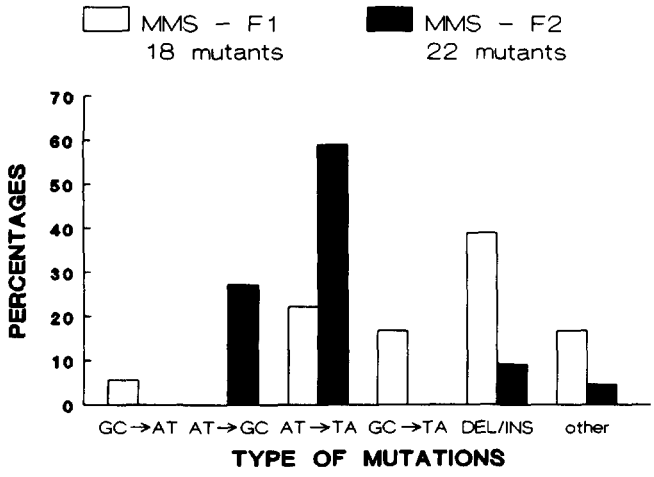

FIGURE 2.—The mutation spectra of transmissible male viable mutations induced by MMS separated in F_1 and F_2 mutants.

F_2 mutants arise as a result of a delay in mutation fixation. It is striking that all except two intralocus deletions induced by MMS were found among F_1 mutants. In addition, there was a significant increase of AT → GC transitions and of AT → TA transversions among the F_2 compared to the F_1 mutants (Table 3, Figure 2). The increase in the AT → TA transversions can be explained by the increase in time available for the formation of apurinic sites from 3-methyladenine. There was, however, not any GC → TA transversion in the F_2-spectrum, which would be expected from depurination of 7-methylguanine. The F_1 spectrum, however, contained three GC → TA transversions compared to four AT → TA transversions. Therefore, we suggest that the AT → TA and GC → TA transversions in the F_1 are caused by a combination of spontaneous and enzymatic hydrolysis of 3- and 7-methylated purine adducts in such a way that the GC → TA base-pair substitutions are mainly caused by spontaneous formation of AP-sites from the 7-methylguanine lesions and the AT → TA transversions by enzymatic formation of AP-sites from the 3-methyladenine lesions. Although the half-life of 7-methylguanine is approximately 5 times longer than that of 3-methyladenine, the *N*7-position is attacked by MMS 10 times more frequently than the *N*3-position (BERANEK 1990). The majority of base substitutions in the F_2 spectrum most likely arise from enzymatic depurinaton. This could mean that also Drosophila contains glycosylases which obviously remove the 3-adenine methylated bases much more efficiently than the 7-guanine methylated bases.

The appearance of a significant number of AT → GC transitions in the F_2 spectrum by MMS was an unexpected observation. The AT → GC transitions found among the F_1 and F_2 mutants in the ENU spectrum could be due to direct mispairing of the O^4-ethylthymine adduct with guanine (PRESTON, SINGER and LOEB 1986). Since this adduct is not produced by MMS, there must be another origin of the AT → GC transitions found with MMS. GENTIL *et al.* (1990) showed that an abasic site located opposite a guanine in mammalian cells leads to the insertion at random of any of the three bases. On basis of the sequence data recovered from the F_1-mutants, Drosophila, like bacteria, seems to have preferential insertion for an adenine opposite an abasic site. Another possibility for the occurrence of AT → GC transitions is that: "Later" cell divisions may be less precise concerning the "A-rule" of KUNKEL (1984), which could mean that instead of an adenine a cytosine or guanine is inserted opposite a noninformative site. However, the fact that there is, compared to the F_1, in the F_2 spectrum, no clear increase in AT → CG transversions makes this explanation rather unlikely. Remaining as a further mechanism is the presence of some transmethylase activity, a hypothesis to be tested in further studies.

The work described in this paper has been supported by a grant from the European Communities, Environmental Research Programme EEG EV4V00414. We wish to thank SHARDA BHAGWANDIEN-BISOEN, INEKE BOGERD, JOSE VAN HELTEN-ZONNEVELD and CORRIE VAN VEEN for their dedicated technical assistance. We thank I. AQUIRREZABALAGA for his technical assistance in the molecular analysis and are very grateful to J. EEKEN for his technical advice with the cytological analysis.

LITERATURE CITED

ABRAHAMSON, S., F. E. WÜRGLER, C. DE JONGH and H. U. MEYER, 1980 How many loci on the X-chromosome of *Drosophila melanogaster* can mutate to recessive lethals? Environ. Mutagen. **2:** 447–453.

ALBERTINI, A. M., M. HOFER, M. P. CALOS and J. H. MILLER, 1982 On the formation of spontaneous deletions: the importance of short sequence homologies in the generation of large deletions. Cell **29:** 319–328.

BATZER, M. A., B. TEDESCHI, N. G. FOSSETT, A. TUCKER, G. KILROY, P. ARBOUR and W. R. LEE, 1988 Spectra of molecular changes induced in DNA of Drosophila spermatozoa by 1-ethyl-1-nitrosourea and X-rays. Mutat. Res. **199:** 255–268

BENYAJATI, C., A. R. PLACE and W. SOFER, 1983 Formaldehyde mutagenesis in Drosophila. Molecular analysis of *Adh*-negative mutants. Mutat. Res. **111:** 1–7.

BERANEK, D. T., 1990 Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. Mutat. Res. **231:** 11–30.

BREIMER, L. H., J. NALBANTOGLU and M. MEUTH, 1986 Structure and sequence of mutations induced by ionizing radiation at selectable loci in Chinese hamster ovary cells. J. Mol. Biol. **192:** 669–674.

BURNS, P. A., A. J. E. GORDON and B. W. GLICKMAN, 1988 Mutational specificity of *N*-methyl-*N*-nitrosourea in the *lacI* gene of *Escherichia coli*. Carcinogenesis **9:** 1607–1610.

CHAUDHRY, M. A., and M. FOX, 1990 Methylmethane-sulphonate and X-ray-induced mutations in the Chinese hamster *hprt* gene: mRNA phenotyping using polymerase chain reactions. Mutagenesis **5:** 497–504.

DUBRIDGE, R. B., P. TANG, H. CHAO HSIA, P. M. LEONG, J. H. MILLER and M. P. CALOS, 1987 Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol. Cell. Biol. **7:** 379–387.

ECKERT, K. A., C. A. INGLE, D. K. KLINEDINST and N. R. DRINKWATER, 1988 Molecular analysis of mutations induced in human cells by *N*-ethyl-*N*-nitrosourea. Mol. Carcinog. **1:** 50–56.

FULLILOVE, S. L., A. G. JACOBSON and F. R. TURNER, 1978 Embryonic development-descriptive, pp. 105–227 in *The Genetics and Biology of Drosophila*, Vol. 2C, edited by M. ASHBURNER and T. R. F. WRIGHT. Academic Press, New York.

GENTIL, A., G. RENAULT, C. MADZAK, A. MARGOT, J. B. CABRAL-NETO, J. J. VASSEUR, B. RAYNER, J. L. IMBACH and A. SARASIN, 1990 Mutagenic properties of a unique abasic site in mammalian cells. Biochem. Biophys. Res. Commun. **173:** 704–710.

GRAY, M., A. CHARPENTIER, K. WALSH, P. WU and W. BENDER, 1991 Mapping point mutations in the Drosophila *rosy* locus using denaturing gradient gel blots. Genetics **127:** 139–149.

HALL, J. A., H. BRÉSIL and R. MONTESANO, 1985 O^6-Alkylguanine DNA alkyltransferase activity in monkey, human and rat liver. Carcinogenesis **6:** 209–211.

HORSFALL, M. J., M. J. ZEILMAKER, G. R. MOHN and B. W. GLICKMAN, 1989 Mutational specificities of environmental carcinogens in the *lacI* gene of *Escherichia coli*. A host-mediated approach to *N*-nitroso-*N*,*N*-dimethylamine and endogenous mutagenesis *in vivo*. Mol. Carcinog. **2:** 1–9.

HORSFALL, M. J., A. J. E. GORDON, P. A. BURNS, M. ZIELENSKA, G. M. E. VAN DER VLIET and B. W. GLICKMAN, 1990 Mutational specificity of alkylating agents and the influence of DNA repair. Environ. Mol. Mutagen. **15:** 107–122.

KUNKEL, T. A., 1984 Mutational specificity of depurination. Proc. Natl. Acad. Sci. USA **81:** 1494–1498.

LARSON, K., J. SAHM, R. SHENKAR and B. STRAUSS, 1985 Methylation-induced blocks to *in vitro* DNA replication. Mutat. Res. **150:** 77–84.

LAWLEY, P. D., 1974 Some chemical aspects of dose-response relationships in alkylation mutagenesis. Mutat. Res. **23:** 283–295.

LEFEVRE, G., JR., 1967 Sterility, chromosome breakage, X-ray-induced mutation rates and detected mutation frequencies in *Drosophila melanogaster*, Genetics **55:** 263–276.

LEFEVRE, G., JR., 1969 The excentricity of *vermilion* deficiencies in *Drosophila melanogaster*. Genetics **63:** 589–600.

LINDSLEY, D. L., and E. H. GRELL, 1967 *Genetic Variations of Drosophila melanogaster*. Carnegie Inst. Wash. Publ. 627.

LOEB, L. A., and B. D. PRESTON, 1986 Mutagenesis by apurinic/apyrimidinic sites. Annu. Rev. Genet. **20:** 201–230.

LOECHLER, E. L., C. L. GREEN and J. M. ESSIGMANN, 1984 *In vivo* mutagenesis by O^6-methylguanine built into a unique site in a viral genome. Proc. Natl. Acad. Sci. USA **81:** 6271–6275.

LOMONACO, M. B., W. R. LEE and S. H. CHANG, 1987 Identification of an X-ray induced deletion mutant flanked by direct repeats. Nucleic Acids Res. **15:** 7641.

MATZUDA, Y, I. TOBARI, M. MAEMORI and N. SEKI, 1989 Mechanism of chromosome aberration induction in the mouse egg fertilized with sperm recovered from postmeiotic germ cells treated with methyl methanesulfonate. Mutat. Res. **214:** 165–180.

NALBANTOGLU, J., D. HARTLEY, G. PHAER, G. TEAR and M. MEUTH, 1986 Spontaneous deletion formation at the *aprt* locus of hamster cells: the presence of short sequence homologies and dyad symmetries at deletion termini. EMBO J. **5:** 1199–1204.

NALBANTOGLU, J., G. PHAER and M. MEUTH, 1987 DNA sequence analysis of spontaneous mutations at the *aprt* locus of hamster cells. Mol. Cell. Biol. **7:** 1445–1449.

NATARAJAN, A. T., J. W. I. M. SIMONS, E. W. VOGEL and A. A. VAN ZEELAND, 1984 Relationship between cell killing, chromosomal aberrations, sister-chromatid exchanges and point mutations induced by monofunctional alkylating agents in Chinese hamster cells. A correlation with different ethylation products in DNA. Mutat. Res. **128:** 31–40.

OSTERMAN-GOLKAR, S., L. EHRENBERG and C. A. WACHTMEISTER, 1970 Reaction kinetics and biological action in barley of mono-functional methanesulphonic esters. Radiat. Bot. **10:** 303–327.

PARÁDI, E., E. W. VOGEL and E. SZILÁGYI, 1983 Effect of storage and dose on MMS-induced deletions. Complementation analysis of X-chromosomal recessive lethals in the *zeste-white* and *maroon-like* regions of *Drosophila melanogaster*. Mutat. Res. **111:** 145–159.

PARDUE, M. L., 1986 *In situ* hybridization to DNA of chromosomes and nuclei, pp. 111–138 in *Drosophila: A Practical Approach*, edited by D. B. ROBERTS. Oxford University Press, New York.

PASTINK, A., C. VREEKEN, M. J. M. NIVARD, L. L. SEARLES and E. W. VOGEL, 1989 Sequence analysis of *N*-ethyl-*N*-nitrosourea-induced *vermilion* mutations in *Drosophila melanogaster*. Genetics **123:** 123–129.

PASTINK, A., C. VREEKEN, E. W. VOGEL and J. C. J. EEKEN, 1990 Mutations induced at the *white* and *vermilion* loci in *Drosophila melanogaster*. Mutat. Res. **231:** 63–71.

PASTINK, A., E. HEEMSKERK, M. J. M. NIVARD, C. J. VAN VLIET and E. W. VOGEL, 1991 Mutational specificity of ethyl methanesulfonate in excision-repair-proficient and deficient strains of *Drosophila melanogaster*. Mol. Gen. Genet. **229:** 213–218.

PEGG, A. E., D. SCICCHITANO and M. E. DOLAN, 1984 Comparison of the rates of repair of O^6-alkylguanines in DNA by rat liver and bacterial O^6-alkylguanine-DNA alkyltransferase. Cancer Res. **44:** 3806–3811.

PRESTON, B. D., B. SINGER and L. A. LOEB, 1986 Mutagenic potential of O^4-methylthymine *in vivo* determined by an enzymatic approach to site-specific mutagenesis. Proc. Natl. Acad. Sci. USA **83:** 8501–8505.

RICHARDSON, K. K., F. C. RICHARDSON, R. M. CROSBY, J. A. SWENBERG and T. R. SKOPEK, 1987 DNA base changes and alkylation following *in vivo* exposure of *Escherichia coli* to *N*-methyl-*N*-nitrosourea or *N*-ethyl-*N*-nitrosourea. Proc. Natl. Acad. Sci. USA **84:** 344–348.

SAGHER, D., and B. STRAUSS, 1983 Insertion of nucleotides opposite apurinic/apyrimidinic sites in deoxyribonucleic acid during *in vitro* synthesis: uniqueness of adenine nucleotides. Biochemistry **22:** 4518–4526.

SCHAAPER, R. M., B. N. DANFORTH and B. W. GLICKMAN, 1986 Mechanisms of spontaneous mutagenesis: analysis of the spectrum of spontaneous mutation in the *Escherchia coli lacI* gene. J. Mol. Biol. **189:** 273–284.

SCHAAPER, R. M., T. A. KUNKEL and L. A. LOEB, 1983 Infidelity of DNA synthesis associated with bypass of apurinic sites. Proc. Natl. Acad. Sci. USA **80:** 487–491.

SCHALET, A. P., 1986 The distribution of and complementation relationships between spontaneous X-linked recessive lethal mutations recovered from crossing long-term laboratory stocks of *Drosophila melanogaster*. Mutat. Res. **163:** 115–144.

SEARLES, L. L., R. S. RUTH, A. PRET, R. A. FRIDELL and A. J. ALI, 1990 Structure and transcription of the *Drosophila melanogaster vermilion* gene and several mutant alleles. Mol. Cell. Biol. **10:** 1423–1431.

SEED, B., 1983 Purification of genomic sequences from bacteriophage libraries by recombination and selection *in vivo*. Nucleic Acids Res. **11:** 2427–2445.

SINGER, B., and D. GRUNBERGER, 1983 *Molecular Biology of Mutagens and Carcinogens*, pp. 1–347. Plenum Press, New York.

VOGEL, E. W., 1986 *O*-Alkylation in DNA does not correlate with the formation of chromosome breakage events in *Drosophila melanogaster*. Mutat. Res. **162:** 201–213.

VOGEL, E., and A. T. NATARAJAN, 1979a The relation between reaction kinetics and mutagenic action of mono-functional alkylating agents in higher eukaryotic systems. I. Recessive

lethal mutations and translocations in Drosophila. Mutat. Res. **62:** 51–100.

VOGEL, E., and A. T. NATARAJAN, 1979b The relation between reaction kinetics and mutagenic action of mono-functional alkylating agents in higher eukaryotic systems. II. Total and partial sex-chromosome loss in Drosophila. Mutat. Res. **62:** 101–123.

VOGEL, E. W., and A. T. NATARAJAN, 1982 The relation between reaction kinetics and mutagenic action of mono-functional alkylating agents in higher eukaryotic systems. III. Interspecies comparisons, pp. 295–336 in *Chemical Mutagens*, Vol. VII, edited by A. HOLLAENDER and F. J. DE SERRES. Plenum, New York.

VOGEL, E. W., M. J. M. NIVARD, C. A. RAAYMAKERS-JANSEN VERPLANKE, A. A. VAN ZEELAND and J. A. ZIJLSTRA, 1986 Alkylation-induced mutagenesis in higher eukaryotic systems: significance of DNA modifications and DNA repair with regard to genetic endpoints, pp. 219–228 in *Genetic Toxicology of Environmental Chemicals, Part A: Basic Principles and Mechanisms of Action*. Alan R. Liss, New York.

ZIELENSKA, M., D. BERANEK and J. B. GUTTENPLAN, 1988 Different mutational profiles induced by *N*-nitroso-*N*-ethylurea: effects of dose and error-prone DNA repair and correlations with DNA adducts. Environ. Mol. Mutagen. **11:** 473–484.

ZIELENSKA, M., M. J. HORSFALL and B. W. GLICKMAN, 1989 The dissimilar mutational consequences of S_N1 and S_N2 DNA alkylation pathways: clues from the mutational specificity of dimethylsulphate in the *lacI* gene of *Escherichia coli*. Mutagenesis **4:** 230–234.

Communicating editor: A. CHOVNICK