Sequence Analysis of *N*-Ethyl-*N*-Nitrosourea-Induced *vermilion* Mutations in *Drosophila melanogaster*

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ABSTRACT

The mutational specificity of *N*-ethyl-*N*-nitrosourea (ENU) was determined in *Drosophila melanogaster* using the *vermilion* locus as a target gene. 25 mutants (16 F_1 and 9 F_2 mutants) were cloned and sequenced. Only base-pair changes were observed; three of the mutants represented double base substitutions. Transition mutations were the most prominent sequence change: 61% were GC→AT and 18% AT→GC substitutions. Both sequence changes can be explained by the miscoding properties of the modified guanine and thymine bases. A strong bias of neighboring bases on the occurrence of the GC→AT transitions or a strand preference of both types of transition mutations was not observed. The spectrum of ENU mutations in *D. melanogaster* includes a significant fraction (21%) of transversion mutations. Our data indicate that like in other prokaryotic and eukaryotic systems also in *D. melanogaster* the O^6-ethylguanine adduct is the most prominent premutational lesion after ENU treatment. The strong contribution of the O^6-ethylguanine adduct to the mutagenicity of ENU possibly explains the absence of distinct differences between the type of mutations observed in the F_1 and F_2 mutants. Although the latter arise later during development, the spectrum of mosaic mutations is also dominated by GC→AT transition mutations.

MOST alkylating agents (AAs) are known to induce mutations, chromosomal aberrations and tumors in a variety of prokaryotic and eukaryotic organisms. Treatment of DNA *in vivo* or *in vitro* with these agents results in a diverse set of adducts (SINGER and GRUNBERGER 1983). The relative distribution of these adducts in the DNA is dependent on the nucleophilic selectivity of the compound used and has an effect on the type of mutations recovered. Previously, it was shown that there is a strong correlation between the relative extent of alkylation at the base oxygens and the induction of mutations (measured as recessive lethal mutations). With compounds acting more extensively at the ring-nitrogen atoms in the DNA, a relatively high chromosome breakage effect was observed in addition to mutations (VOGEL and NATARAJAN 1979, 1982; NATARAJAN *et al.* 1984). Analysis of ethyl methanesulfonate (EMS)- and *N*-ethyl-*N*-nitrosourea (ENU)-induced mutations at the *adh*, *white*, *RpII215* and *ry* loci in *Drosophila melanogaster* by blot-hybridization, indicated that most of the mutations induced by these monofunctional ethylating agents are caused by base-pair changes or deletions smaller than 50–100 bp (COTÉ *et al.* 1986; LACY, EISENBERG and OSGOOD 1986; BATZER *et al.* 1988; PASTINK, VREEKEN and VOGEL 1988). The objective of this study has been to characterize by sequence analysis alkylation-induced mutations and to gain information on the contribution of specific adducts to the type of mutations finally recovered.

Against this background, we selected a small group of mono-functional alkylating carcinogens, differing in their Swain-Scott constant's (SWAIN and SCOTT 1953), for sequence analysis of alkylation-induced mutations. This paper reports the analysis of ENU-induced mutations at the *vermilion* locus. ENU is a potent carcinogen and has the ability to ethylate various sites in the DNA. The major alkylation sites are the O^6 and *N*-7 positions of guanine, the O^2 positions of cytosine and thymine, the *N*-3 position of adenine and the phosphodiester groups in the DNA backbone. One of the minor alkylation sites is the O^4 position of thymine (BERANEK, WEIS and SWENSON 1980, and references cited therein). ENU represents the first chemical carcinogen of a whole series of monofunctional AAs whose mutational spectra are analyzed by DNA sequence characterization in our laboratory. Comparative studies of this type may eventually contribute to a better understanding of the sequence of events leading from the initial DNA adduct to the final genetic alteration.

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The wild-type eye color of *D. melanogaster* is a composite of red and brown pigments. Mutations at the *vermilion* locus result in a bright red eye color. The *vermilion* locus has been studied extensively at the genetic and biochemical level (LINDSLEY and GRELL 1968; O'BRIEN and MACINTYRE 1978). This gene codes for the enzyme tryptophan oxygenase (BAGLIONI 1960; BAILLIE and CHOVNICK 1971), which catalyzes the first step in the synthesis of the brown eye pigment of the fly. Because tryptophan oxygenase is not cell autonomous, *i.e.*, diffuses through the whole body, most mosaic mutations will result in a wild-type eye color. The gene has been cloned and the nucleotide sequence is also known (SEARLES and VOELKER 1986; WALKER, HOWELLS and TEARLE 1986; L. L. SEARLES, unpublished results). The *vermilion* gene has a size of about 2 kb and codes for a mRNA of 1.3 kb (see Figure 1 for further details). Due to its small size the *vermilion* locus is very suitable for the sequence analysis of mutations due to base-pair changes and small deletions. A potential disadvantage may be seen in the fact that many large multilocus deletions are not recovered due to the proximity of a haplo-insufficient female fertility gene to the right of the *vermilion* gene (LEFEVRE 1967, 1969).

The mutational specificity of ENU has been determined in *Escherichia coli* and in human cells using episomal genes as targets (RICHARDSON *et al.* 1987; ECKERT *et al.* 1988). Only base-pair changes were observed, the majority being transition mutations. Our results obtained by germ cell analysis using an endogenous chromosomal locus as a target gene, are in good agreement with the other studies.

MATERIALS AND METHODS

Bacterial strains and media: All strains are *E. coli* K-12 derivatives. XS127 is Δ(*lac-proXIII*), *gyrA*, *thi*, *rpoB argE*, [*F'*, *laci*^{q}*ZΔM15*, *proAB*, *traD36*], [p3, *kan*, *amp*^{am}, *tet*^{am}], LG75 is *sup*^{o}, *lacZ*^{am} (LEVINSON, SILVER and SEED 1984; SHEN and HUANG 1986). LC medium contains per liter 10 g bactotryptone, 5 g yeast extract and 8 g NaCl. The constitution of NZCYM medium has been described by MANIATIS, FRITSCH and SAMBROOK (1982).

Isolation of mutants: For the induction of *vermilion* mutants, *bw* males (derived from wild-type Berlin K) were placed in glass vials containing 8 layers of glass microfiber paper (Whatmann GF/A) soaked with 0.9 ml of a 1mM ENU solution in 33 mM sodium phosphate (pH 6.0); 5% sucrose. After 24 hr exposure, males were mated in bottles to *In(1)sc*^{S1L}*sc*^{8R} *In(1)dl-49*, *y sc*^{S1}*sc*^{8} *v*; *bw* virgin females, at a sex ratio of 1:1. Since the combination of *v* and *bw* yields white eyes, the marker *bw* was introduced in the strains, in order to facilitate the identification of *vermilion* mutants. Fractionation of the progeny into broods was undertaken: after 2 days (brood 1A), males were remated to virgin females for 3 days (brood 2A). Females of brood 1A were transferred three times to yield broods 1B, 1C + 1D. The same procedure was used for obtaining broods 2B and 2C. Total length of breeding periods (in days) was 2-3-3-4 for brood 1 and 3-3-4 for brood 2. Of 47 mutant females transmitting a *vermilion* phenotype to the next generation, 40 strains homozygous for *vermilion* could be built up (Table 1). In addition one mutant (#156), which was female sterile, was kept over a *C(1)DX* chromosome.

DNA isolation: About 0.5–1 g flies were frozen in liquid nitrogen and grounded to a fine powder in a mortar in the presence of liquid nitrogen. The powder was resuspended in 15 ml of ice-cold homogenization buffer (0.1 M NaCl; 30 mM Tris-HCl (pH 8.0); 10 mM EDTA; 10 mM 2-mercaptoethanol; 15 mM spermine; 15 mM spermidine; 0.5% Triton X-100) and homogenized with a glass pestle. The homogenate was centrifuged for 10 min at 4000 × *g* and the pellet resuspended in 15 ml extraction buffer (0.1 M NaCl; 0.1 M Tris-HCl (pH 8.4); 20 mM EDTA) and centrifuged again. The pellet was resuspended in 10 ml extraction buffer, proteinase K and SDS were added to final concentrations of 100 μg/ml and 1%, respectively, and incubated at 50° for 1 hr. The mixture was cooled to room temperature and 0.2 volume of 8 M potassium acetate was added. After 30 min on ice the DNA solution was extracted twice with chloroform. Nucleic acids were precipitated with 1 volume of ethanol and dissolved in 10 mM Tris-HCl (pH 7.5); 1 mM EDTA (TE buffer). The DNA samples were treated successively with pancreatic RNase (20 μg/ml) and proteinase K (50 μg/ml) and further purified by fenol-chloroform-isoamylalcohol and chloroform-isoamylalcohol extractions. After ethanol precipitation the DNA was dissolved in TE buffer.

Cloning of *vermilion* alleles: The rapid cloning and sequencing procedure used, is based on the recombination screening method, originally developed by B. SEED (SEED 1983; LUTZ *et al.* 1987). Fly DNA was digested with *Hin*dIII and *Xba*I, producing a 5.2-kb *Hin*dIII fragment containing the *vermilion* gene (Figure 1). Digestion with *Xba*I, which does not cut inside the 5.2-kb fragment, was included to enhance the enrichment after fractionation on a 0.8% agarose gel. DNA fragments ranging in size between 4.4 kb and 6.7 kb were eluted from the gel and ligated into PDJ11, an *A*^{am}*B*^{am} lambda vector (P. DE JONG, unpublished results). About 100 ng fly DNA and 800 ng PDJ11 DNA, digested with *Hin*dIII and treated with calf intestine phosphatase, were ligated in a volume of 10 μl. After packaging *in vitro* the primary library was amplified by plate lysis. About 15,000–20,000 recombinant phages were used to infect XS127 harboring the probe plasmid pMv2. In addition to *vermilion* sequences a *supF* gene is located on this plasmid, allowing the suppression of the amber mutations of the lambda vector (Figure 2). Plating cells were grown in NZCYM medium containing 0.2% maltose, ampicillin and tetracycline. After infection, the cells were plated on 10-cm NZCYM plates in soft agar containing ampicillin. Basic phage techniques were performed as described by MANIATIS, FRITSCH and SAMBROOK (1982).

Homologous recombination between phages containing the 5.2-kb *vermilion* fragment results in the incorporation of the probe-plasmid in the phage genome. Plate stocks were harvested and used for infection of LG75 to select for phage-plasmid cointegrates. After infection, cells were plated in soft agar containing X-Gal (0.65 mg/ml) and isopropyl thiogalactoside (0.40 mg/ml). Phage-plasmid cointegrates will give rise to blue plaques. By plaque-hybridization it was shown that nearly all blue plaques indeed contain *vermilion* sequences. Single blue plaques were picked and plated again on LG75.

Sequencing of *vermilion* alleles: About one-third of a resuspended blue plaque was used to infect LG75 and plated in top agarose on 10 cm NZCYM agarose plates. Phage DNA was isolated from plate lysates as described by DAVIS

TABLE 1

Isolation of *vermilion* mutants after treatment of postmeiotic male germ cells with 1.0 mM ENU for 24 hr

Brood	Storage period (in days)	Offspring scored for *v* in F_1 or F_2	No. transmissible *v* mutants: Male viable	No. transmissible *v* mutants: Male lethal	Transmissible total	Frequency ($\times 10^{-4}$)	Sequence analysis	Sterile[a]	Not transmitted[a]
1A	No storage	F_1 31,254	3	1	4	1.3	1	1	
		F_2 7,188	1		1	(1.4)	1		
1B	0–3	F_1 45,388	7	1	8	1.8	4	3	1
		F_2 6,202	2	1	3	4.8	2		
1C	3–6	F_1 40,054	2		2	0.5	2		
		F_2 2,706	1		1	(3.7)			
1D	6–10	F_1 17,763	1		1	(0.6)			
		F_2 968	1		1	(10.3)			
2A	No storage	F_1 49,089	7	2	9	1.8	4	2	1
		F_2 6,131	2		2	3.3	2		
2B	0–3	F_1 34,915	5		5	1.4	3	2	
		F_2 3,435	2		2	5.8	2		
2C	0–7	F_1 26,719	5	1	6	2.2	2		2
		F_2 985	2		2	20.3	2		
Total		F_1 245,182	30	5	35	1.4	16	8	4
		F_2 27,615	11	1	12	4.4	9		

[a] Not included in calculation of frequency.

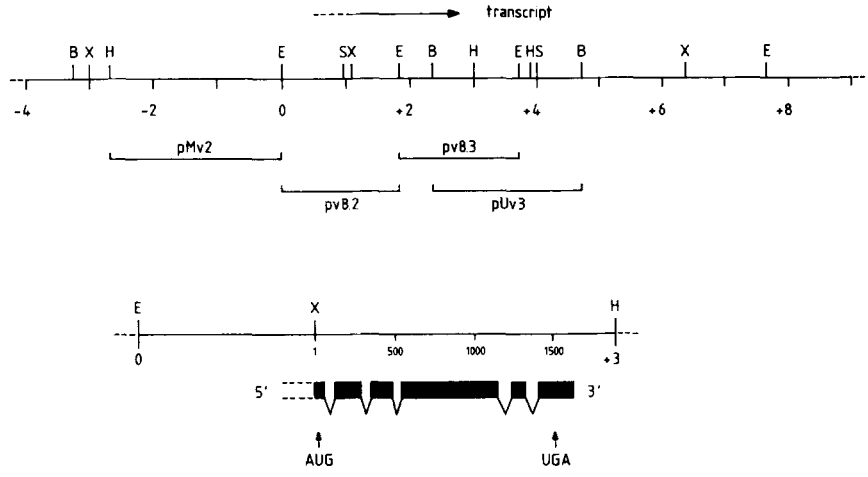

FIGURE 1.—Restriction enzyme map of the *vermilion* gene. Restriction enzyme sites were taken from SEARLES and VOELKER (1986). The coordinate scale is in kilobases. *Sst*I sites were mapped only in the region between coordinates 0.0 and +4. The bottom part shows an enlargement of the *Eco*RI-*Hin*dIII fragment between coordinates 0.0 and 3.0. The nucleotide sequence of this region has been determined by L. L. SEARLES (unpublished results). The nucleotide numbering starts at the adenine within the *Xho*I site at coordinate +1.1. The intron-exon structure is depicted as determined by L. L. SEARLES (unpublished results). The start of transcription is not yet known, as is indicated by the dotted area. The protein coding region is located between nucleotide 32 and 1523. The nucleotide sequence of mutant *vermilion* alleles was determined for the region between nucleotide 1 and 1700 using a set of 10 oligonucleotide primers. Restriction endonuclease sites are *Sst*I (S), *Hin*dIII (H), *Bam*HI (B), *Eco*RI (E) and *Xho*I(X).

et al. (1980). Digestion with *Hin*dIII results in a 7.2-kb fragment containing the protein coding region of the *vermilion* gene and upstream sequences as well as plasmid sequences (Figure 2). This fragment is circularized at a low DNA concentration (50–100 ng in 10 μl) and used to transform XS127. Transformants were selected on NZCYM plates containing ampicillin and tetracycline. For the isolation of single-stranded plasmid DNA, a single colony was grown in LC medium containing ampicillin. During early log-phase (OD_{600} of 0.15–0.20) RV-1 helper phage (LEVINSON, SILVER and SEED 1984) was added at a multiplicity of infection of 25. The culture was grown for 14–18 hr at 37° with good aeration. Single-stranded DNA was isolated as described before (PASTINK *et al.* 1988). Sequencing was carried out using the dideoxy chain termination method (SANGER, NICKLEN and COULSON 1977). A series of 10 oligonucleotide primers was used for the sequence determination of the region between nucleotide 1 and 1700 (Figure 1).

Blot-hybridization: Genomic DNAs were digested with *Hin*dIII-*Xba*I and with *Eco*RI-*Sst*I, electrophoresed, transferred to nylon membranes and hybridized at 65°C as described before (PASTINK *et al.* 1987). pv8.2 and pUv3 were used as probes (Figure 1).

RESULTS

Inspection of 245,182 F_1 female flies resulted in the isolation of 35 transmissible *vermilion* mutants, yielding a frequency of 1.4 mutants per 10,000 offspring (Table 1). This rate is 14 times above the background frequency of about 1 mutant in 100,000 flies (Schalet, personal communication). Because the *vermilion* gene product is not cell autonomous, *i.e.*, diffuses through the whole body of the flies, mosaic mutants cannot be recognized in the first generation. Therefore, a total

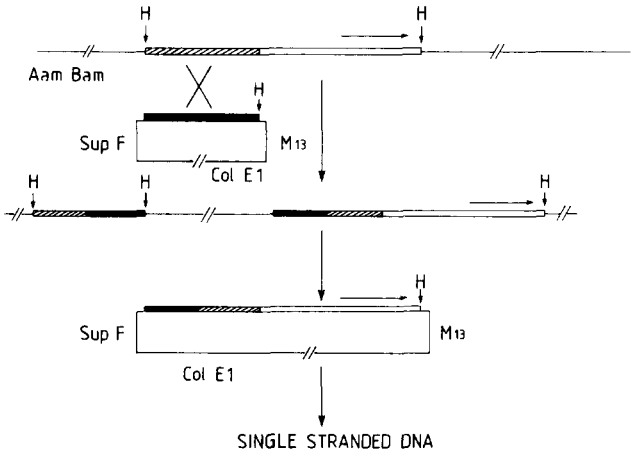

FIGURE 2.—Scheme for the cloning of *vermilion* alleles. Homologous recombination between phages containing the 5.2-kb *vermilion* fragment and the probe plasmid produces a phage-plasmid cointegrate. The transcribed region of the *vermilion* gene within the 5.2 kb *Hin*dIII fragment is indicated by the arrow. The 2.1-kb *Hin*dIII-*Eco*RI fragment between coordinates − 2.1 and 0.0, was used for the recombination (Figure 1). This fragment was, after filling in the *Hin*dIII site with polIk, cloned in *Sma*I-*Eco*RI digested pIC20H (MARSH, ERFLE and WYLER 1984) and subsequently cloned as an *Xba*I-*Hin*dIII fragment into pMLS13. In this way the *Hin*dIII site on the left side of the probe fragment is removed and a new one is introduced at the right side, permitting the recovery of the *vermilion* gene on a plasmid after *Hin*dIII digestion of phage-plasmid cointegrates and circularization. The pMLS13 vector is identical to pSDL13 (LEVINSON, SILVER and SEED 1984), except for the second *Hin*dIII site outside the multi-cloning sequence, and contains a *supF* gene capable of suppressing the amber mutations of the lambda vector and also an M13 origin of replication permitting the isolation single-stranded DNA. The restriction enzyme abbreviation is: H, *Hin*dIII.

of 27,615 F_1 nonmutant females were individually crossed to F_1 males, giving 12 flies with an aberrant eye color in the F_2. The resulting frequency of 4.4×10^{-4} is three times higher than the frequency of *vermilion* mutants in the F_1. Table 1 shows that only 6 out of 47 *v* mutants were male-lethal, and that another eight F_1 females of a mutant phenotype were sterile. The high proportion of transmissible viable *v* mutants is consistent with the view that ENU is a strong point mutagen. With the cross-linking agent hexamethylphosphoramide, for instance, more than 60% of all F_1 mutant phenotypes were either sterile or male-lethal (M. J. M. NIVARD and E. W. VOGEL, unpublished observation). Thus, 40 strains homozygous for *v* were constructed, whereas one (#156) female sterile mutant was kept over a *C(1)DX* chromosome. Most of the mosaic mutants have arisen after a few rounds of cell division. Some of these F_2 mutants were included in our analysis because a delay in mutation fixation may result in a different type of genetic alteration. From 16 F_1 and 9 F_2 mutants DNA was isolated and used for further analysis. Blot-hybridization indicated that no major genomic alteration had occurred in any of these mutants suggesting that mutation by ENU involves only base-pair changes or very small rearrangements. As mentioned before, large deletions also affecting adjacent loci are not recovered at the *vermilion* locus. However, the analysis of ENU-induced mutations at the *white* locus also indicated that base-pair substitutions are the predominant type of genetic alteration produced by an agent like ENU (PASTINK, VREEKEN and VOGEL 1988). ENU-induced large deletions would have been recovered at the *white* locus (PASTINK *et al.* 1987). Aside from this reasoning, analysis of DNA alterations of 20 F_1 *v* mutants induced by the methylating agent methylmethanesulfonate revealed 30% intralocus deletions (M. J. M. NIVARD, unpublished observation), clearly demonstrating that the *vermilion* system can detect this type of event. To determine the specific sequence alterations of the ENU-induced *vermilion* mutants, the sequence of the region between nucleotide 1 and 1700 was determined. This region contains the protein coding part of the gene, but not the start of transcription (Figure 1). The wild-type *vermilion* allel from the Berlin K strain was independently cloned and sequenced two times, and compared with the sequence determined for the Canton S allel (L. L. SEARLES, unpublished results). Nucleotide differences were observed at nucleotides 233 (G→C), 327 (T→A), 425 (C→T), 1374 (A→C), 1648 (C→T) and an extra T between 1390 and 1400. At the level of the protein no differences were observed between both wild-type strains. The results of the analysis of 25 ENU-induced mutants are summarized in Table 2. Single base substitutions were observed in 22 mutants. In three mutants (107, 153 and 155) two base-pair changes had occurred. In mutant 155 the second change is located within intron sequences. In both mutant 106 and 177 the base change results in an altered splice site. All other base substitutions give rise to amino acid changes or result in a nonsense codon. The GC→AT transition at position 1322 was observed in two mutants, namely 158 and 167. It is very unlikely that the duplicates are contaminants because the mutants were isolated from different experiments. The types of base pair changes observed are summarized in Table 3. Approximately 61% of the sequence changes are GC→AT transitions, whereas 18% are AT→GC transition mutations. The spectrum of ENU-induced mutations in *D. melanogaster* also includes a significant fraction of transversion mutations (21%). In three mutants the alteration is an AT→TA change. The other transversion mutations were observed in one mutant each (Table 3).

DISCUSSION

The spectrum of mutations induced by ENU in the germline of *D. melanogaster* is dominated by GC→AT (61%) and AT→GC (18%) transition mutations. In

TABLE 2

ENU mutants

Mutants	Brood	Position	Change	Amino acid	Target sequence 5′-3′
106	2A	58	GC → AT	Splice	AACGG G TGAGC
107	2A	988	AT → GC	his → arg	AGTGC A CGATG
		1053	AT → GC	ile → val	TCATG A TCACC
111	1A(F_2)	1430	GC → AT	asp → asn	TTCTG G ATCTG
114	2B(F_2)	838	GC → AT	trp → UAG	CTTCT G GGCCA
152	2A(F_2)	468	GC → AT	val → met	ACCGA G TGGTT
155	2A	585	GC → AT	asp → asn	CGCTA G ACTTC
		498	AT → TA	Intron	CTTTC T GAATC
159	2A	285	GC → AT	his → tyr	TCACG C ACCAG
101	2B	624	GC → AT	gly → ser	CATCT G GTTTT
103	1B(F_2)	365	GC → AT	trp → UGA	CTTTG G TTCAA
108	1B	1014	GC → AT	arg → cys	GGGAT C GCCGG
113	1B	372	GC → AT	gln → UAG	TCAAG C AGATC
150	2B	1331	GC → AT	ser → asn	TCTCA G GTGAT
151	1B(F_2)	1048	AT → CG	ile → ser	AGCCA T CATGA
153	1B	912	AT → TA	lys → UAG	TGGAG A AGGCG
		936	GC → CG	asp → his	TCATG G ACATT
156	2A	224	GC → TA	gln → his	GCCCA G TGTAT
158[a]	2B	1322	GC → AT	ser → phe	GCGCT C CACTC
161	1B	207	AT → TA	lys → UAA	TGGAC A AACTG
164	2C	1126	GC → AT	ser → leu	CGACT C GTTAA
166	2C(F_2)	1316	AT → GC	leu → pro	ATATC T GCGCT
167[a]	2B(F_2)	1322	GC → AT	ser → phe	GCGCT C CACTC
168	2C(F_2)	796	AT → GC	leu → pro	ATGGC T GGAGA
171	1C	1017	GC → AT	arg → trp	ATCGC C GGTTT
174	1C	1117	AT → GC	asp → gly	CATGG A CATCG
177	2A(F_2)	351	GC → AT	Splice	CCACA G CCTAC
185	2C	1122	GC → AT	asp → asn	ACATC G ACTCG

[a] Isolated from different experiments.

TABLE 3

Classification of ENU-induced *vermilion* mutants

G:C → A:T	Transition	17
A:T → G:C	Transition	5
A:T → T:A	Transversion	3
A:T → C:G	Transversion	1
G:C → C:G	Transversion	1
G:C → T:A	Transversion	1

five out of seven ENU-induced *ry* mutants also transitions were observed (SUNG LEE *et al.* 1987). Similar results also have been obtained with human cells and *E. coli* (ECKERT *et al.* 1988; RICHARDSON *et al.* 1987; ZIELENSKA, BERANEK and GUTTENPLAN 1988). In *E. coli* the percentage of transitions is even higher. Both the GC→AT and AT→GC transitions most probably result from unrepaired O^6-guanine and O^4-thymine adducts which lead to mispairing with thymine and guanine, respectively, during replication (see for example LOECHLER, GREEN and ESSIGMANN 1984; PRESTON, SINGER and LOEB 1986). In addition to the transition mutations a significant fraction (21%) are transversions. The ENU spectrum in human cells also includes a significant portion of transversions (ECKERT *et al.* 1988). In contrast, ENU induces hardly any transversion mutations in *E. coli* (RICHARDSON *et al.* 1987). The transversion mutations in *D. melanogaster* and in human cells can be explained by misrepair and/or miscoding properties of the ethylated bases. Misinsertion opposite apyrimidinic or apurinic (AP) sites can also be a mutagenic event (LOEB and PRESTON 1986). The formation of AP sites, predominantly resulting in the insertion of an adenine opposite the AP site, may be of importance in eukaryotic organisms. This is a time-dependent process (SIGNER and GRUNBERGER 1983), which could be one reason why in *E. coli* or in other short term systems, where the time interval between carcinogen interaction with DNA and mutation fixation is short, mutation induction correlates best with *O*-alkylation. Thus the majority of the potentially labile adducts may not yield AP sites during the time for which the bacteria are at risk for mutagenesis (WISEMAN *et al.* 1986). Another reason for the induction of transversion mutations in eukaryotic cells may be the absence of specific glycosylases, as has been suggested by ECKERT *et al.* (1988), to explain the relatively high number of AT→TA transversions in human cells after ENU treatment. It is striking that three of the six transversions we observed, are in mutant genes with two base changes. The double mutations may be the result of a nonrandom distribution of ethyl adducts in the DNA (NEHLS

and RAJEWSKY 1985). However, it cannot be excluded that some of the base-pair changes we observed are the consequence of a reduced fidelity of the replicating machinery induced by the presence of ethyl adducts in the DNA.

Both F_1 and F_2 mutants were isolated and analyzed in this study, in order to see if mutations which were manifested later during development would differ from those fixed earlier. Although the numbers are small, there is no indication of a difference in the spectrum between F_1 and F_2 mutants (Table 2). There is also no apparent difference in the nature of sequence changes in mutants isolated from different broods. In all cases transition mutations are the predominant type of base changes. It appears that a possible increase in the number of AP sites in time does not noticeably alter the spectrum of mutations, probably because such an effect is superimposed by the relative high proportion of miscoding O^6-guanine and O^4-thymine adducts in the DNA.

Examination of the DNA sequences surrounding the GC→AT transitions did not reveal a strong preference for guanine at the 5′-side of the mutated base, like it has been found for other alkylating agents (DUBRIDGE *et al.* 1987; BURNS, GORDON and GLICKMAN 1987, 1988). Neither did we observe a strand preference of the GC→AT and AT→GC transitions. A strong sequence context or strand bias was also absent in the ENU spectrum in human cells (ECKERT *et al.* 1988). On the other hand, in *E. coli* RICHARDSON *et al.* (1987) observed a strong bias in neighboring bases as well as in the strand specificity. It may well be that the size and/or the type of the target gene used influences the distribution of the alterations.

The results described in this paper demonstrate the applicability of the *vermilion* locus in *D. melanogaster* for molecular analysis of genetic alterations induced by carcinogenic compounds in a whole animal.

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