Jpn. J. Cancer Res. 88, 461–467, May 1997

Comparative Study on Germ Cell Mutation Induced by Urethane (Ethyl Carbamate) Gas and X-rays in *Drosophila melanogaster*

Taisei Nomura[1,3] and Nobuo Kurokawa[2]

[1]*Department of Radiation Biology and* [2]*Hospital Pharmacy, Faculty of Medicine, Osaka University, 2-2 Yamada-Oka, Suita 565*

Although its mutagenicity has not been confirmed in mouse germ cells, urethane (ethyl carbamate) gas induces a significant increase of X-linked recessive lethal mutations in the germ cells of *Drosophila melanogaster*. The mutation frequency increased as the exposure time was changed from 3.5 to 5.5 h. Mutations were also induced by X-rays (20 to 40 Gy) and *N*-methyl-*N*-nitrosourea (MNU) (0.06 to 0.10%). However, no significant increase of chromosomal changes (partial loss of the Y chromosome, total loss of X or Y, and translocations) was produced by urethane, although these were readily induced by X-rays. There were large and significant increases in chromosomal changes caused by X-rays (20 Gy) compared to urethane (5.5 h) or MNU (0.06%). In contrast, there were no substantial differences among these three treatments as regards recessive lethal mutations. Urethane-induced DNA lesions detected as recessive lethals appear to be intragenic mutations. Complementation analysis with 15 reference single-site loci (cistrons) in the *zeste-white* region of the X chromosome revealed that 29 of 723 urethane-induced recessive lethals were located in the *zeste-white* region and all were restricted to a single locus. However, among 28 of 890 X-ray-induced lethals, 2 were non-complementary to 2 or 3 adjacent loci, indicating deletions encompassing 2 or 3 loci. In addition, 3 of these lethal chromosomes included mutations outside the *zeste-white* region. Another difference between urethane and X-rays was in the distribution of mutation sites. Urethane-induced mutations were strikingly non-random with two hot spots at *zw-1* and *zw-2*, whereas the distribution of X-ray-induced mutations was more nearly random.

Key words: Urethane (ethyl carbamate) — X-rays — Germ cell mutation — Complementation analysis — *Drosophila melanogaster*

Until 1976, urethane (ethyl carbamate) had long been used as a sedative, hypnotic, anesthetic, and solvent in more than 200 commonly used drugs for humans. It has been used widely in pesticides and cosmetics.[1-3] It is also contained in fermented beverages and foods[4,5] and has proved to be useful in formulating everyday products under many patents.[2] However, potent carcinogenic and teratogenic effects of urethane have been reported in various experimental animals,[1,6] and vinyl carbamate is suspected to be a proximate form to produce genotoxicity.[7-12] Furthermore, a significant increase in somatic mutations was produced in mice by increasing doses of urethane,[13] and preconceptional treatment with urethane resulted in a significant increase of tumors and anomalies in the offspring,[14,15] although germ cell mutations were not detected in mice by the dominant lethal[15-17] and 7 specific loci methods.[18] In *Drosophila melanogaster*, however, recessive lethal mutations were induced in germ cells by oral administration of urethane.[12,19,20]

Urethane is volatile, and sublimed pure urethane molecules are highly mutagenic in the germ cells of *Drosophila melanogaster*[2] and also induce high incidences of cancer,[21] malformations and chromosome aberrations[22] in mice. In the present study, we have confirmed the mutagenicity of sublimed urethane gas in *Drosophila* germ cells. To determine the nature of the mutations, we conducted a complementation analysis of recessive lethal mutations in the *zeste-white* region of the X chromosome and compared them with those induced by X-rays.

MATERIALS AND METHODS

Examination of chromosomal changes and specific locus and recessive lethal mutations One gram of powdered urethane (ethyl carbamate, CAS No. 51-79-6, Wako Pure Chemical Ind. Ltd., Osaka) wrapped in cotton was covered with cheesecloth and pressed to the bottom of a 22 mm vial. Fifty male flies of the Oster strain, $y^2\ w^i\ ct^6\ f/sc^8\ Y\ B^s$, were placed in the vial, which was tightly stoppered with a wad of cotton. Although the flies were quickly anesthetized by spontaneously sublimed urethane gas, they continued to take up the gas.[2] In contrast, flies fed urethane-containing medium were quickly anesthetized and did not eat further. Males were exposed to urethane gas for 3.5 or 5.5 h in the vial at 22°C. Concurrent controls were treated similarly without urethane. At 24 h after urethane or X-ray treatment, 50 treated males were mass-mated with 25 virgin females of the constitu-

[3] To whom correspondence should be addressed.

tion *y* sc^{s1} *In49* sc^{8}; *dp bw*; *st* p^{p} in a half-pint bottle containing cornmeal-molasses medium. After 48 h, treated males were discarded and the inseminated females were transferred to a fresh half-pint bottle. Consequently, the spermatozoa stage was treated. After 48 h, females were again transferred to a fresh bottle and discarded after 48 h. After emergence, F_1 males were examined genetically for partial loss of the Y chromosome and complete loss of the X or Y chromosome.[23] The F_1 males and females were also examined for specific locus mutations at the *dp* locus. F_1 males were placed individually in vials with 2 or 3 *y* sc^{s1} *In49* sc^{8}; *dp bw*; *st* p^{p} virgin females to detect *T (2; 3)*, *T (Y; 2)* and *T (Y; 3)* translocations in the F_2 progeny.[24] For the detection of X-linked recessive lethal mutations, F_1 virgin females were collected and mated individually in vials with 2–3 Muller-5 males of the constitution *In (1)* sc^{s1} sc^{8} w^{a} *B*. X-linked recessive lethal mutations were detected by the Muller-5 technique and confirmed at and after the F_2 generation.[2,20]

As positive controls, Oster males were fed on Kleenex tissue saturated with 0.06 or 0.1% MNU (*N*-methyl *N*-nitrosourea; Sigma, St. Louis, MO) in 1% sucrose solution in 0.1 *M* sodium phosphate buffer (pH 5.8) for 24 h or exposed to 20 or 40 Gy of X-rays at a dose rate of 1.26 Gy/min. For X-irradiation, a Toshiba Model KC-18-2A (Toshiba Medical Co., Ltd., Tokyo) was used, operating at 20 mA and 180 kVp with a filter of 1 mm of aluminum at a distance of 45.0 cm. Doses were measured by a Condenser R-meter Model 570 (Victoreen Instr. Div., Cleveland, OH). For descriptions of stocks and mutants, see Lindsley and Grell.[25]

Complementation analysis Experimental procedures for complementation analysis of urethane- and X-ray-induced recessive lethal mutations are given in Fig. 1.[26–28] Oster males (described above) were treated with ure-

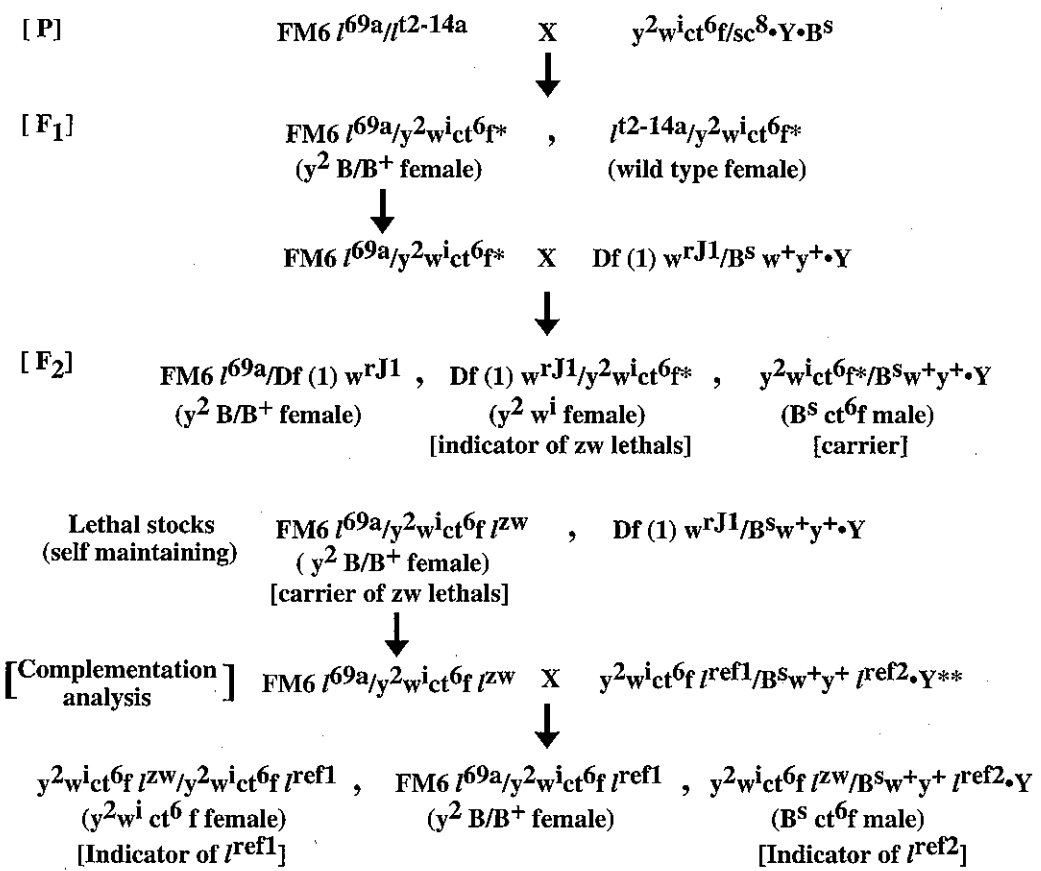

Fig. 1. Mating scheme to identify urethane- or X-ray-induced recessive lethal mutations in the *zeste-white* region. *, Treated X chromosome. **, Df 202/DfL126, Df 7 and Df 304 were used for prescreening before the use of reference single-site lethal stocks. l^{zw} indicates urethane- or X-ray-induced recessive lethal mutation in the *zeste-white* region, and l^{ref} indicates reference single-site lethal stock in the *zeste-white* region.

Table I. Tester Stocks for the Complementation Analysis in the *zeste-white* Region of the X Chromosome

Abbreviation	Genotype
69a/tko/L26 (gt)	*FM6 l (1)^{69a}/y^2 w^i ct^6 f l (1)^{tko}/B^s w$^+$y$^+$ l (1)^{gt}·Y*
69a/zw-8/L34 (zw-1)	*FM6 l (1)^{69a}/y^2 w^i ct^6 f l (1)$^{zw\text{-}8}$/B^s w$^+$y$^+$ l (1)$^{zw\text{-}1}$·Y*
69a/zw-10/L61 (zw-4)	*FM6 l (1)^{69a}/y^2 w^i ct^6 f l (1)$^{zw\text{-}10}$/B^s w$^+$y$^+$ l (1)$^{zw\text{-}4}$·Y*
69a/zw-2/L38 (zw-13)	*FM6 l (1)^{69a}/y^2 w^i ct^6 f l (1)$^{zw\text{-}2}$/B^s w$^+$y$^+$ l (1)$^{zw\text{-}13}$·Y*
69a/zw-6/L79 (zw-3)	*FM6 l (1)^{69a}/y^2 w^i ct^6 f l (1)$^{zw\text{-}6}$/B^s w$^+$y$^+$ l (1)$^{zw\text{-}3}$·Y*
69a/zw-7/L4 (zw-12)	*FM6 l (1)^{69a}/y^2 w^i ct^6 f l (1)$^{zw\text{-}7}$/B^s w$^+$y$^+$ l (1)$^{zw\text{-}12}$·Y*
69a/zw-5/L1 (zw-11)	*FM6 l (1)^{69a}/y^2 w^i ct^6 f l (1)$^{zw\text{-}5}$/B^s w$^+$y$^+$ l (1)$^{zw\text{-}11}$·Y*
69a/zw-9/L1 (zw-11)	*FM6 l (1)^{69a}/y^2 w^i ct^6 f l (1)$^{zw\text{-}9}$/B^s w$^+$y$^+$ l (1)$^{zw\text{-}11}$·Y*
69a/Df 202/Df-L126	*FM6 l (1)^{69a}/y^2 w^i ct^6 f Df (1) 202/B^s w$^+$y$^+$ Df (1) L126·Y*
69a/Df 7/B^{s}w$^+$y$^+$·Y	*FM6 l (1)^{69a}/y^2 w^i ct^6 f Df (1) 7/B^s w$^+$y$^+$·Y*
69a/Df 304/B^{s}w$^+$y$^+$·Y	*FM6 l (1)^{69a}/y^2 w^i ct^6 f Df (1) 304/B^s w$^+$y$^+$·Y*
69a/Df (1)w^{rJ1}/B^{s}w$^+$y$^+$·Y	*FM6 l (1)^{69a}/y^2 w$^-$ spl ec sn^3 Df (1) w^{rJ1}/B^s w$^+$y$^+$·Y*
69a/$l^{t2\text{-}14a}$/y$^+$·Y·mal$^+$	*FM6 l (1)^{69a}/l (1)$^{t2\text{-}14a}$/y$^+$·Y·mal$^+$*

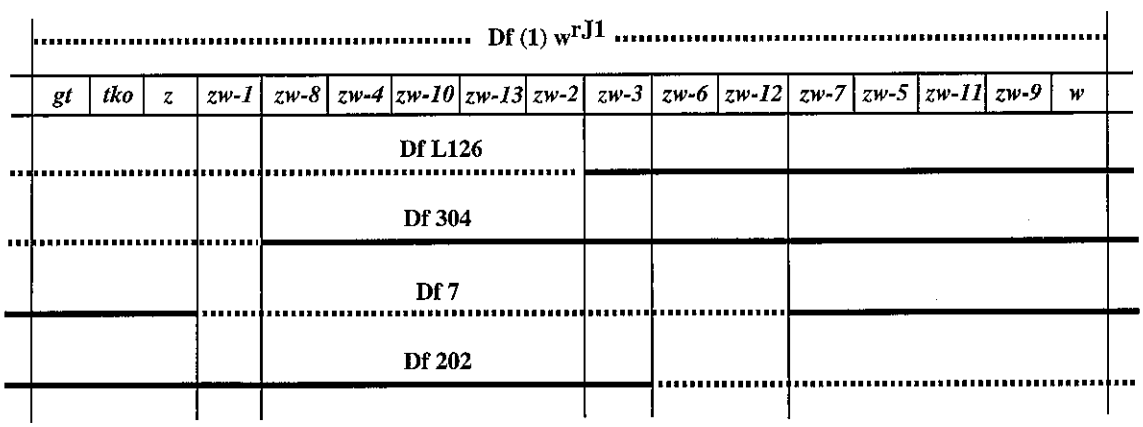

Fig. 2. The extent of deletions in the *zeste-white* regions. Dotted lines indicate the deleted regions.

thane gas for 5.5 h or with 20 Gy of X-rays, and 24 h after treatment males were mated for 48 h with virgin females heterozygous for *FM6, l (1) 69a* and *l (1) t2-14a*. After the mating period, the males were discarded and the females were placed in fresh bottles for an additional 48 h for egg laying. Because of the presence of *l (1) 69a* in the *FM6* chromosome and *l (1) t2-14a* in the other X chromosome, no male progeny is expected from the mating. Consequently, *y^2 B/B$^+$* females [*FM6 l (1) 69a/ y^2 w^i ct^6 f*] are automatically virgin and these females were mated individually with 2–3 *Df (1) w^{rJ1}/B^s w$^+$ y$^+$·Y* males to detect recessive lethal mutations in the F_2 progeny. An F_2 vial without *y^2 w* females [*Df (1) w^{rJ1}/ y^2 w^i ct^6 f*] was assumed to represent a stock with a recessive lethal mutation in the *zeste-white* region. Each of the recessive lethal mutations in the *zeste-white* region was maintained by mating *FM6 l (1) 69a/y^2 w^i ct^6 f l^{zw}* females to *y^2 w^i ct^6 f l^{zw}/B^s w$^+$ y$^+$·Y* males, where *l^{zw}* denotes urethane- or X-ray-induced recessive lethal mutation in the *zeste-white* region. This mating scheme is self-maintaining and at the same time provides a means of checking the lethality of a particular mutant allele. Recessive lethal mutant stocks obtained previously with the use of different strains (FM7, Muller-5, Oregon R)[2, 20] and for this study were also mated with *Df (1) w^{rJ1}/B^s w$^+$y$^+$·Y* and subjected to complementation analysis.

Virgin females *FM6 l (1) 69a/y^2 w^i ct^6 f l^{zw}* were prescreened with triethylene melamine-induced recessive lethal strains *Df (1) L126, Df (1) 304, Df (1) 7 and Df (1) 202*, which are deficient in a part of the *zeste-white* region (Table I and Fig. 2), to define subregions where urethane- or X-ray-induced mutations are located. After that, these recessive lethal mutants were mated with genetically known single-site recessive lethal stocks at the 15 loci (cistrons): *gt, tko, zw-1, zw-8, zw-4, zw-10, zw-13, zw-2, zw-3, zw-6, zw-12, zw-7, zw-5, zw-11 and zw-9*[27, 28] to

Table II. Induction of Total Loss of the X or Y Chromosome, Partial Loss of Y, Specific Locus Mutation, X-linked Recessive Lethal Mutation, and Translocation by Urethane Gas and X-rays in *Drosophila melanogaster* (%)

Treatment	Loss of X or Y	Partial loss of Y[a)]	Specific locus mutations at *dp* locus	Recessive lethal mutations	Y; II, Y; III, and II; III translocations
Control	0/1605 (0.00)	1/1605 (0.06)	0/3232 (0.00)	1/1424 (0.07)	0/460 (0.00)
Urethane gas (3.5 h)	0/934 (0.00)	0/934 (0.00)	0/2030 (0.00)	22/711[c)] (3.09)	0/462 (0.00)
Urethane gas (5.5 h)	5/2935 (1)[b)] (0.17)	1/2935 (3)[b)] (0.03)	1/6141 (0.02)	58/1203[c)] (4.82)	1/502 (0.20)
MNU (0.06%)	1/849 (2)[b)] (0.12)	0/849 (0.00)	0/1188 (0.00)	9/144[c)] (6.25)	0/329 (0.00)
MNU (0.1%)	5/2112 (2)[b)] (0.24)	11/2112[d)] (0.52)	0/4662 (2)[b)] (0.00)	70/761[c)] (9.20)	0/688 (0.00)
X-rays (20 Gy)	16/1629[c)] (0.98)	10/1629[d)] (0.61)	4/2961 (0.14)	38/748[c)] (5.08)	37/646[c)] (5.73)
X-rays (40 Gy)	8/732[c)] (1.09)	14/732 (1)[b, c)] (1.91)	4/1609[d)] (0.25)	24/204[c)] (11.8)	89/661[c)] (13.5)

a) Loss of y^+ and B^s, which are terminal markers on the long and short arms of Y chromosome, respectively.
b) Mosaic.
c, d) χ^2 test with Yates' correction yielded *P* values of 0.001 (*c*) and 0.05 (*d*) when compared to the control group.

determine the nature of the urethane- and X-ray-induced mutations. All mutant strains for complementation analysis were provided by Dr. J. K. Lim.

RESULTS

Genetic effects of urethane Sublimed urethane gas induced a significant number of recessive lethal mutations, and the incidence increased with increasing time of urethane gas inhalation ($P \ll 0.001$) (Table II). The incidence of urethane gas-induced recessive lethal mutations in the present study did not differ substantially from previous results with Oregon R males and FM 7 females.[2)] X-rays and the potent carcinogen MNU also induced a very high incidence of recessive lethal mutations ($P \ll 0.001$). The incidences increased with higher dose and concentration. However, chromosomal changes (partial loss of the Y chromosome, total loss of X or Y, and translocations) and specific locus mutations were not significantly induced by 3.5 h inhalation of urethane gas or by the lower concentration (0.06%) of MNU. Longer inhalation of urethane gas (5.5 h) and higher concentration of MNU (0.1%) induced more chromosomal changes, but the frequencies were not significantly higher than those of the controls, except for the total incidence of chromosomal changes (partial loss of Y and total loss of X or Y) by 0.1% MNU (16/2112, 0.76%, $P < 0.01$), while much higher incidences of these defects were induced by X-rays ($P \ll 0.001$). There were large and significant differences between the incidence of these two chromosomal changes from 20 Gy of X-rays and those from 5.5 h inhalation of urethane gas and 0.06% MNU treatment ($P < 0.001$ vs. urethane and $P < 0.002$ vs. MNU), although there were no substantial differences in the incidence of recessive lethal mutations from the X-ray (20 Gy), urethane (5.5 h), and MNU (0.06%) treatments. Much higher differences were observed in the incidence of translocations between X-ray exposure and urethane gas and MNU treatments ($P \ll 0.001$). Specific locus mutations at the *dp* locus were rarely induced by urethane and MNU. The incidence was also low with X-rays, but was significantly increased by 40 Gy of X-rays. Such differences in chromosomal changes (break, aneuploidy, and translocation) between urethane and X-rays had also been suggested by our previous studies with different strains of *Drosophila melanogaster*.[2, 20)]

Complementation maps of urethane- and X-ray-induced mutations Among 723 urethane- and 890 X-ray-induced recessive lethal mutations of X chromosomes, 29 and 28 respectively were located in the *Df(1) w^{rJ1}* region and were mapped in the *zeste-white* region. As shown in Fig. 3, all of 29 urethane-induced mutations in the *zeste-white* region were restricted to a single locus, i.e., single-site mutations. However, 2 of 28 X-ray-induced mutations were non-complementary to 2 and 3 adjacent loci, *gt–zw-1* and *zw-4–zw-10*, indicating deletions encompassing 2

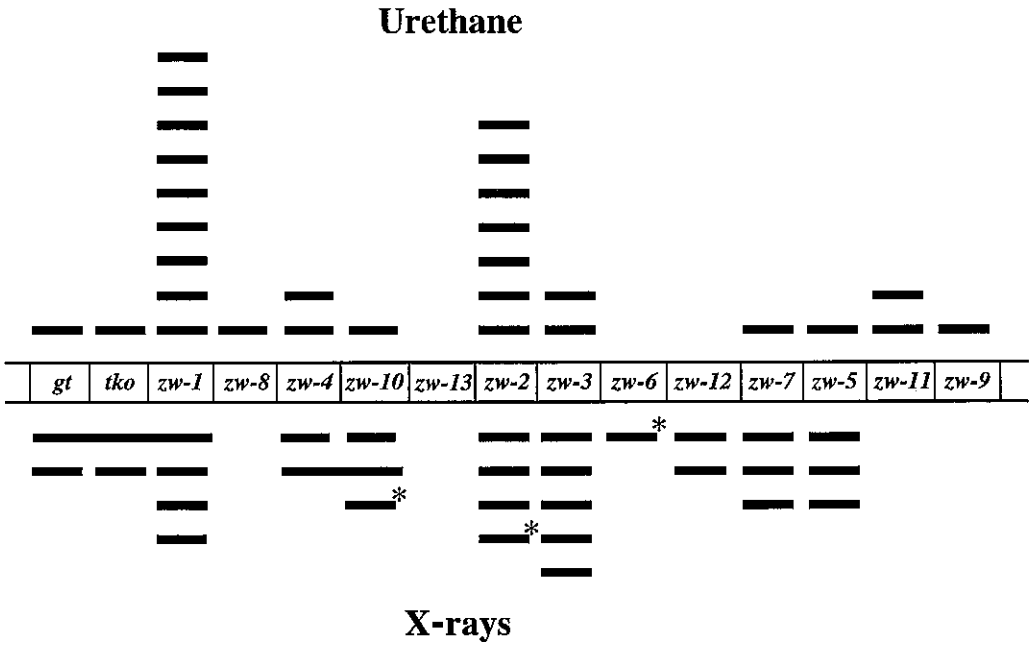

Fig. 3. Complementation maps of urethane- and X-ray-induced recessive lethal mutations in the *zeste-white* region. *, Multiple mutations which carried other mutations outside the *zeste-white* region.

and 3 loci, and 3 recessive lethal mutants at *zw-2, zw-6* and *zw-10* loci carried other mutations outside the *zeste-white* region of the X chromosome. The distribution of urethane-induced mutations was non-random; there were 2 sharp hot spots at the *zw-1* and *zw-2* loci. The results are compatible with those in the cases of ethyl methanesulfonate[27] and methyl methanesulfonate.[28] In contrast, the distribution of X-ray-induced mutations was more nearly random than that of urethane-induced mutations, although mutations were more often seen at *zw-3, zw-1* and *zw-2* loci, and also at *zw-5, zw-7* and *zw-10* loci. However, no mutations were induced at *zw-13* by either urethane or X-rays in the present study.

DISCUSSION

In *Drosophila melanogaster*, Oster[19] reported that feeding of standard *Drosophila* medium containing 2.5 *M* urethane (LD_{50} dose) induced chromosomal changes and recessive lethal mutations. In our study,[20] however, one twenty-fifth the concentration (0.1 *M*) of urethane on tissue paper was the maximum non-anesthetizing dose and induced 3 times as many recessive lethal mutations, but a lower incidence (1/4) of chromosomal changes. In the process of preparing urethane-containing *Drosophila* medium, urethane may have been sublimed and/or decomposed to other forms by heating and reacting with other components of the medium in the previous study.[19] Vogt[29] injected urethane solution into adult *Drosophila* males and obtained significant yields of X-linked recessive lethal mutations. The incidence was only 5.5 times that in untreated controls. Furthermore, an extensive study by Fahmy and Fahmy[30] showed only very weak mutagenicity by the same Muller-5 technique. In contrast, the high volatility of urethane[2] permitted treating *Drosophila* more effectively without any interruption of feeding by sleeping, because flies, although anesthetized, nevertheless take up urethane gas by breathing. Furthermore, they take up sublimed pure urethane molecules, not residual carbamates.[2] Even by inhalation of urethane gas, no significant increase of dominant lethals (data not shown) and chromosomal changes was observed in *Drosophila* germ cells in the present study, though all of these defects are easily induced by X-rays or γ-rays. Similar results had been reported in the mouse germ cells with X-rays and urethane.[15, 31] Although urethane gas induced chromosomal aberrations in mouse bone marrow cells and embryos, most were chromatid types.[22] Some mutagens that did not induce translocations in *Drosophila* by the usual method have turned out to induce translocations when treated sperm were stored in females,[24, 32, 33] but no increased incidence of translocations was observed even if the treated sperm were stored for more than 10 days.[2] However, the spontaneous rate of trans-

locations in *Drosophila* is so low that even 1 or 2 translocations may represent a slight increase.[19, 20, 32] Failure of the induction of translocations by urethane may be the major cause of the lack of dominant lethals in *Drosophila* and mice,[15–17, 31] and urethane-induced DNA lesions responsible for the induction of recessive lethal mutations in *Drosophila* and also tumors in mouse offspring[14, 15, 31] may not necessarily be large chromosomal changes, but rather may be point or intragenic mutations. In fact, complementation analysis revealed that all of the urethane-induced mutations were located within a single locus, suggesting base substitutions or deletion of some bases, while X-rays produced some larger deletions and multiple mutations. Although urethane can induce somatic crossing-over (recombination) between *flr* and *mwh* in *Drosophila* larvae heterozygous at these two wing loci,[34] recombination hardly occurs in *Drosophila* male germ cells, particularly in the recessive lethal mutations detected by the Muller-5 technique.[25] Several reports have also described K-*ras* mutation, mostly AT-to-GC transition and AT-to-TA transversion at the second base of codon 61, in urethane-induced tumors.[35–37]

The remaining discrepancy is the negative result for induction of specific locus mutations in the mouse germ cells irrespective of treated stages.[18] However, parental exposure to urethane at postmeiotic stages has induced tumors and anomalies in the offspring[14, 15, 31] and most *in vivo* somatic toxicities, i.e., somatic mutation, malformation and cancer, have been well demonstrated in mice and other animals[6, 13, 21, 22, 34] in spite of negative genotoxicities in cultured mammalian cells.[38] In particular, most gene loci examined by the specific locus test in mouse germ cells and the somatic spot test in the mouse embryo are the same.[13] Metabolic activation specific for urethane may be deficient or low in the mouse germ cells when compared with that in the somatic cells, or strain differences, i.e., genotypic differences, may cause the difference in sensitivity to urethane-induced mutation and cancer.[34, 39, 40] Alternatively, mouse germ cells, especially spermatogonia, may have powerful repair capacity for urethane-induced DNA damage.[18, 31, 40]

ACKNOWLEDGMENTS

I would like to thank Professors J. F. Crow and J. K. Lim for their advice, Mrs. H. Ryo for her help, and JFC for his critical review and editorial polishing of the manuscript.

(Received December 20, 1996/Accepted February 21, 1997)

REFERENCES

1) Nomura, T. Urethan (ethyl carbamate) as a cosolvent of drugs commonly used in humans. *Cancer Res.*, **35**, 2895–2899 (1975).
2) Nomura, T. Potent mutagenicity of urethan (ethyl carbamate) gas in *Drosophila melanogaster*. *Cancer Res.*, **39**, 4224–4227 (1979).
3) Crout, J. R. Drug product containing urethan. Opportunity for hearing on proposal to withdraw approval of new drug application for profenil injection. United States Federal Registry Document No. 76–5599 (1976).
4) Ough, C. S. Ethyl carbamate in fermented beverages and foods. I. Naturally occurring ethyl carbamate. *Agric. Food Chem.*, **24**, 323–328 (1976).
5) Christoph, N., Schmidt, A. and Hidenbrand, K. Untersuchungen zur bildung und zum destillationsverhalten von äthylcarbamat bei der branntweinherstellung. *Alkoholindustrie*, **15**, 347–354 (1986).
6) Nomura, T. An analysis of the changing urethan response of the developing mouse embryo in relation to mortality, malformation and neoplasm. *Cancer Res.*, **34**, 2217–2231 (1974).
7) Allen, J. W., Langenbach, R., Nesnow, S., Sasseville, K., Leavitt, S., Campbell, J., Brock, K. and Sharief, Y. Comparative genotoxicity studies of ethyl carbamate and related chemicals: further support for vinyl carbamate as a proximate carcinogenic metabolite. *Carcinogenesis*, **3**, 1437–1441 (1982).
8) Crebelli, R., Bellincampi, D., Conti, G., Conti, L., Morpurgo, G. and Carere, A. A comparative study on selected chemical carcinogens for chromosome malsegregation, mitotic crossing-over and forward mutation induction in *Aspergillus nidulans*. *Mutat. Res.*, **172**, 139–149 (1986).
9) Leithauser, M. T., Liem, A., Stewart, B. C., Miller, E. C. and Miller, J. M. 1, N^6-Ethenoadenosine formation, mutagenicity and murine tumor induction as indicators of the generation of an electrophilic epoxide metabolite of the closely related carcinogens ethyl carbamate (urethane) and vinyl carbamate. *Carcinogenesis*, **11**, 463–473 (1990).
10) Guengerich, F. P., Kim, D. H. and Iwasaki, M. Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem. Res. Toxicol.*, **4**, 168–179 (1991).
11) Park, K. K., Liem, A., Stewart, B. C. and Miller, J. A. Vinyl carbamate epoxide, a major strong electrophilic, mutagenic and carcinogenic metabolite of vinyl carbamate and ethyl carbamate (urethane). *Carcinogenesis*, **14**, 441–450 (1993).
12) Ballering, L. A. P., Nivard, M. J. M. and Vogel, E. W. Characterization by two-endpoint comparisons of the genetic toxicity profiles of vinyl chloride and related ethenoadduct forming carcinogens in *Drosophila*. *Carcinogenesis*, **17**, 1083–1092 (1996).
13) Nomura, T. Comparative inhibiting effects of methylxan-

thines of urethan-induced tumors, malformations, and presumed somatic mutations in mice. *Cancer Res.*, **43**, 1342–1346 (1983).
14) Nomura, T. Transmission of tumors and malformations to the next generation of mice subsequent to urethane treatment. *Cancer Res.*, **35**, 264–266 (1975).
15) Nomura, T. Parental exposure to X rays and chemicals induces heritable tumours and anomalies in mice. *Nature*, **296**, 575–577 (1982).
16) Bateman, A. J. Mutagenic sensitivity of maturing germ cells in the male mouse. *Heredity*, **12**, 213–232 (1958).
17) Bateman, A. J. A failure to detect any mutagenic action of urethane in the mouse. *Mutat. Res.*, **4**, 710–712 (1967).
18) Russell, L. B., Hunsicker, P. R., Oakberg, E. F., Cummings, C. C. and Schmoyer, R. L. Tests for urethane induction of germ-cell mutations and germ-cell killing in the mouse. *Mutat. Res.*, **188**, 335–342 (1987).
19) Oster, I. I. Interactions between ionizing radiation and chemical mutagens. *Z. Vererbungsl.*, **89**, 1–6 (1958).
20) Nomura, T., Kurokawa, N., Isa, Y., Sakamoto, Y., Kondo, S. and Endo, H. Induction of lymphoreticular neoplasia and malformations by prenatal treatment with 1, 3-di(4-sulfamoylphenyl)triazene in mice. *Carcinogenesis*, **5**, 571–575 (1984).
21) Nomura, T., Hayashi, T., Masuyama, T., Tanaka, S., Nakajima, H., Kurokawa, N. and Isa, Y. Carcinogenicity of sublimed urethane in mice through the respiratory tract. *Jpn. J. Cancer Res.*, **81**, 742–746 (1990).
22) Nomura, T., Tanaka, S., Kurokawa, N., Shibata, K., Nakajima, H., Kurishita, A., Hongyo, T. and Ishii, Y. Cytogenotoxicities of sublimed urethane gas to the mouse embryo. *Mutat. Res.*, **369**, 59–64 (1996).
23) Snyder, L. A. and Oster, I. I. A comparison of genetic changes induced by a monofunctional and a polyfunctional alkylating agents in *Drosophila melanogaster*. *Mutat. Res.*, **I**, 437–445 (1964).
24) Snyder, L. A. Evidence of an essential difference between point mutations and chromosome breaks induced by triethylene melamine in *Drosophila spermatozoa*. *Z. Vererbungsl.*, **94**, 182–189 (1963).
25) Lindsley, D. L. and Grell, E. H. Genetic variation of *Drosophila melanogaster*. *Carnegie Inst. Washington Publ.* No. 627 (1972).
26) Judd, B. H., Shen, M. W. and Kaufman, T. C. The anatomy and function of a segment of the *X* chromosome of *Drosophila melanogaster*. *Genetics*, **71**, 139–156 (1972).
27) Lim, J. K. and Snyder, L. A. Cytogenetic and complementation analyses of recessive lethal mutations induced in the X chromosome of *Drosophila* by three alkylating agents. *Genet. Res., Camb.*, **24**, 1–10 (1974).
28) Liu, C. P. and Lim, J. K. Complementation analysis of methyl methanesulfonate-induced recessive lethal mutations in the *zeste-white* region of the *X* chromosome of *Drosophila melanogaster*. *Genetics*, **79**, 601–611 (1975).
29) Vogt, M. Mutationsauslosung bei *Drosophila* durch Athylurethan. *Experientia (Basel)*, **4**, 68–69 (1948).
30) Fahmy, O. G. and Fahmy, M. J. Gene elimination in carcinogenesis: reinterpretation of the somatic mutation theory. *Cancer Res.*, **30**, 195–205 (1970).
31) Nomura, T. Changed urethan and radiation response of the mouse germ cell to tumor induction. *In* "Tumours of Early Life in Man and Animals," ed. L. Severi, pp. 873–891 (1978). Perugia Univ. Press, Perugia.
32) Auerbach, C. and Kilbey, B. J. Mutation in eukaryotes. *Annu. Rev. Genet.*, **5**, 163–218 (1971).
33) Munoz, E. R. and Mazar Barnett, B. II-III translocations induced by diethyl sulfate in mature sperm of *Drosophila melanogaster*. *Mutat. Res.*, **45**, 355–357 (1977).
34) Frölich, A. and Würgler, F. E. Genotoxicity of ethyl carbamate in the Drosophila wing spot test: dependence on genotype-controlled metabolic capacity. *Mutat. Res.*, **244**, 201–208 (1990).
35) Nuzum, E. O., Malkinson, A. M. and Beer, D. G. Specific Ki-ras codon 61 mutations may determine the development of urethan-induced mouse lung adenomas or adenocarcinomas. *Mol. Carcinog.*, **3**, 287–295 (1990).
36) Ohmori, H., Abe, T., Hirano, H., Murakami, T., Katoh, T., Gotoh, S., Kido, M., Kuroiwa, A., Nomura, T. and Higashi, K. Comparison of Ki-ras gene mutation among simultaneously occurring multiple urethane-induced lung tumors in individual mice. *Carcinogenesis*, **13**, 851–855 (1992).
37) Kawano, R., Nishisaka, T., Takeshima, Y., Yonehara, S. and Inai, K. Role of point mutation of the K-*ras* gene in tumorigenesis of B6C3F$_1$ mouse lung lesions induced by urethane. *Jpn. J. Cancer Res.*, **86**, 802–810 (1995).
38) Sofuni, T., Honma, M., Hayashi, M., Shimada, H., Tanaka, N., Wakuri, S., Awogi, T., Yamamoto, K. I., Nishi, Y. and Nakadate, M. Detection of *in vitro* clastogens and spindle poisons by the mouse lymphoma assay using the microwell method: interim report of an international collaborative study. *Mutagenesis*, **11**, 349–355 (1996).
39) Falconer, D. S. and Bloom, J. L. A genetic study of induced lung-tumours in mice. *Br. J. Cancer*, **16**, 665–685 (1962).
40) Ehling, U. H. Methods to estimate the genetic risk. *In* "Mutations in Man," ed. G. Obe, pp. 292–318 (1984). Springer-Verlag, Berlin, Heidelberg.