*TEMPERATURE-SENSITIVE MUTATIONS IN DROSOPHILA MELANOGASTER, I. RELATIVE FREQUENCIES AMONG γ-RAY AND CHEMICALLY INDUCED SEX-LINKED RECESSIVE LETHALS AND SEMILETHALS**

BY DAVID T. SUZUKI, LEONIE K. PITERNICK, SUSI HAYASHI, MARY TARASOFF, DAVID BAILLIE, AND UDO ERASMUS

DEPARTMENT OF ZOOLOGY, UNIVERSITY OF BRITISH COLUMBIA, VANCOUVER, BRITISH COLUMBIA, CANADA

Communicated by Curt Stern, February 20, 1967

Genetic and biochemical analysis in microorganisms has been greatly facilitated by the use of conditional lethal mutations that die under "restrictive" conditions but survive in a "permissive" environment.[1, 2] One class of conditional lethals, the temperature-sensitive (ts) mutants that result from a single amino acid substitution that renders a polypeptide inactive at high temperatures,[3–5] has been extensively analyzed in *Neurospora*,[6] bacteria,[7] T4 phages,[2, 8] and tobacco mosaic virus.[3, 4] In higher organisms, ts mutants have been detected in *Arabidopsis*,[9] *Paramecium*,[10] and *Drosophila*.[11] The potential usefulness of ts mutants in several types of genetic analyses prompted us to screen for them systematically in *Drosophila melanogaster*. This paper reports the frequencies of ts mutants among sex-linked recessive lethals and semilethals induced by γ-rays, ethyl methanesulfonate, and mitomycin C.

Methods and Materials.—Screening technique: Oregon-R males were collected within 48 hr of eclosion, treated as described below, and then mated separately with three Basc females in vials at room temperature (23° ± 2°C). The males were transferred without etherization to a fresh set of females at 2-day intervals for several consecutive broods. Each set of females was removed from the vials 3 days after transfer of the males in the experiments with mitomycin C and γ-rays. Owing to the poor fertility of males treated with ethyl methanesulfonate, females in these experiments were allowed to lay eggs for up to 5 days after transfer of the males and in some cases were then transferred to a fresh vial. Single F_1 females from each brood of each male were placed together with one or two Basc males at 29° ± 0.5°C. The presence of two or less non-Basc F_2 males in a fertile culture suggested that a lethal or semilethal had been induced on the X chromosome. Replicate cultures of the F_2 of all possible lethals and semilethals were set at 29°C and 17°C. The appearance of at least two non-Basc males at 17°C in a culture that had yielded none in the second 29°C test or of several non-Basc males at 17°C in a stock in which one or two had hatched in the second 29°C test indicated possible temperature sensitivity of the mutant. Mutants which were ts for developmental rate, visible phenotype, or subvitality were not scored as ts in the present data.

After having been maintained in stock for a period of time varying from 1 to 5 months, all of the putative ts mutants in each series (see later section) were again screened for temperature sensitivity. Three vials of each stock were raised at 29°C and three vials at 17°C, and all of the offspring in each vial at each temperature were scored. Three counts were made of each vial at early, mid-, and late hatching times in order to ensure complete recovery of all flies hatched. Progeny of females heterozygous for either the FM-6 or the Basc chromosome and an X chromosome from the Oregon-R stock were scored at 29°C and at 17°C as controls.

Series A and B: Ethyl methanesulfonate (EMS): In preliminary experiments, EMS was used as a mutagen since it induces missense mutations in T4 phages[12] and is known to be highly mutagenic in *Drosophila*.[13] EMS solutions (0.2–2%) were administered by injection in the gonadal area,[14] by feeding through a capillary tube,[15] or feeding for 48 hr on Kleenex tissues saturated with the solution.[16]

Some of the induced lethals and semilethals in each test were found to be temperature-sensitive, although the relative frequency of ts mutants varied with each type of treatment. The results

of the preliminary experiments were combined as Series A. In Series B, males were placed for 24 hr in quarter-pint bottles containing Kleenex tissues saturated with 10 ml of 0.025 *M* EMS dissolved in a 1% sucrose solution and then removed and mated as outlined for five broods.

Series C: γ-Rays: Males were placed in gelatin capsules, irradiated with 4000 r of γ-rays delivered from a 6000-c cobalt[60] therapeutic source at 200 r/min and mated as outlined.

Series D–G: Mitomycin C (MC): Males were fed MC for 48 hr in the manner outlined for Series B. In the initial experiment, concentrations of 100 (Series D), 250 (Series E), and 500 (Series F) μg/ml of MC were used. Only broods 3 and 4 were tested for lethal induction since MC has its maximal effect in these broods when injected.[17] In Series G, males were fed 250 μg/ml of MC and all five successive 2-day broods were analyzed for lethals.

Results.—The results of the preliminary experiments indicated that ts mutants can be recovered and that 6.3 per cent of all EMS-induced lethals and semilethals are ts (Series A, Table 1). Henceforth, "lethals" will include both lethals and semilethals. Only the proportion of ts mutants among the lethals is meaningful since each test included in the total yielded mutants at different frequencies. In the standardized test (Series B), the over-all mutation frequency in the three fertile broods was 62.7 per cent, and 14.5 per cent of these lethals were initially scored as ts. The proportion of ts mutants in each brood is significantly different from each other. However, it cannot be inferred that this does indeed reflect an altered sus-

TABLE 1

NUMBER OF TEMPERATURE-SENSITIVE LETHALS AND SEMILETHALS AMONG OFFSPRING IN EACH BROOD OF MALES TREATED WITH DIFFERENT MUTAGENS

		Brood Number 1			2			3		
Series	Mutagen	No.	l	ts	No.	l	ts	No.	l	ts
A	EMS	2,045	392	22	1,231	240	14	981	142	13
	%		19.2*	5.6†		19.5	5.8		14.4	9.2
B	EMS	642	410	56	553	340	64	281	169	14
	%		63.8	13.7		61.4	18.8		60.1	8.3
C	γ-Rays	1,243	116	3	1,338	88	4	562	40	0
	%		9.3	2.6		6.6	4.5		7.1	0.0
D	MC		Not tested			Not tested		609	25	1
	%								4.1	4.0
E	MC		Not tested			Not tested		813	54	2
	%								6.6	3.7
F	MC		Not tested			Not tested		1,258	45	0
	%								3.6	0.0
G	MC	1,354	4	1	1,201	12	1	1,150	18	1
	%		0.3	25.0		1.0	8.3		1.6	5.5

		Brood Number 4			5			Total		
Series	Mutagen	No.	l	ts	No.	l	ts	No.	l	ts
A	EMS	241	37	2		Not tested		4,498	811	51
	%		15.4	5.4					18.0	6.3
B	EMS		Sterile			Sterile		1,486	919	134
	%								61.8	14.5
C	γ-Rays	65	5	0		Sterile		3,208	249	8
	%		7.7	0.0					7.8	3.2
D	MC	683	10	0		Not tested		1,282	35	1
	%		1.5	0.0					2.7	2.8
E	MC	943	42	2		Not tested		1,756	96	4
	%		4.4	4.8					5.5	4.2
F	MC	1,083	23	0		Not tested		2,341	68	0
	%		2.1	0.0					2.9	0.0
G	MC	1,042	14	0	827	11	1	5,515	59	4
	%		1.3	0.0		1.3	9.1		1.1	6.7

No. = Number of chromosomes tested.
l = Number of sex-linked recessive lethals and semilethals at 29°C.
ts = Number of ts lethals and semilethals.
* Per cent of lethals.
† Per cent of lethals classified as putative ts.

TABLE 2

NUMBER OF LETHAL AND SEMILETHAL MUTATIONS INDUCED BY DIFFERENT MUTAGENS THAT FALL IN EACH ts CLASS

Range of (Wild-Type ♂/Het ♀) Ratio (29°C)	(17°C)	EMS (Series B)	γ-Ray	MC	Class
0.945	0.850				Control (FM-6)
0.971	0.886				Control (Basc/+)
0.0	0.10–0.19	5	2	1	
0.0	0.20–0.49	14	0	4	ts
0.0	0.50–0.90	11	5	2	ts
0.01–0.05	0.30–0.90	20	2	3	ts
0.06–0.09	0.50–0.90	7	0	0	ts
0.10–0.20	0.50–0.90	7	1	0	ts
	Total ts	59	8	9	

ceptibility of different cell stages to ts induction as the brood analysis used does not permit critical separation of different sperm stages.

In Series B, out of a total of 1,870 F_1 females tested, 384 (20.5%) were sterile. This is in contrast to the results in Series A in which fewer than 10 per cent of all F_1 females tested were sterile. The high rate of sterility in Series B as compared to Series A is probably a consequence of the induction of deleterious mutants throughout the genome which affect fertility or viability.

The total frequency of lethals (7.8%) induced by 4000 r of γ-rays (Series C) is similar to that reported by others.[18] On the other hand, frequencies of mutants induced by different concentrations of mitomycin C fluctuated from test to test. The values for the rate of lethal induction by feeding MC (Series D–F) were similar to those obtained after injection of the mutagen[17] and indicated that a concentration of 250 μg/ml (Series E) yields a maximum number. However, in Series G, mutation frequencies were lower than the values obtained in Series E. It is possible that the older solution of MC used in Series G had deteriorated in storage, or the results may indicate a heterogeneity resulting from feeding the mutagen. In all four MC tests, more than 50 per cent of the treated males were sterile by the time brood 5 matings were made, an indication that MC was taken up and has a potent physiological effect. Of all lethals produced by γ-rays and MC, 3.2 per cent and 3.5 per cent, respectively, were scored as ts.

The ts mutants were maintained balanced with either an FM-6 or a Basc chromosome. Therefore, in the retest of all putative ts mutants, progeny from two different kinds of control females were scored at the two temperatures. The frequency of males carrying the FM-6 or Basc chromosomes was greatly depressed at both experimental temperatures; therefore, the ratio of wild-type males to heterozygous females was used as the most reliable index of relative viability of the X chromosome tested. In the controls, heterozygous females and wild-type males only were scored for a total of 5219 and 5272 offspring of FM-6 and 7656 and 3926 offspring of Basc females at 29°C and 17°C, respectively. The ratios of females to males can be seen in Table 2 and are in good agreement with the expected 1:1 ratio at 29°C. There is a significant reduction in the recovery of males at 17°C.

In the retest of all lethals and semilethals initially classified as ts, a minimum of 100 heterozygous females was scored at each temperature for each mutant. The results of the retest of all putative ts mutants obtained in the initial screening and

confirmed once at 29°C can be seen in Table 2. It was arbitrarily decided that mutants completely lethal at 29°C (wild-type males/heterozygous females = 0.0) but yielding at least 20 per cent of the expected number of males at 17°C or room temperature would be classified as ts. Semilethals at 29°C that produced males with a frequency of at least 30 per cent of heterozygous females and three times as many male offspring carrying the ts chromosome at 17°C as at 29°C were also classified as ts. By these criteria, 31 (60.7%) of the 51 Series A ts mutants were confirmed as being temperature-sensitive under the conditions of the retest. Fifty-nine (43.7%) of the 135 ts mutants of Series B were ts. Of the remainder, 14 were lethal at both temperatures, 16 semilethal (less than 10% viability) at 17°C and lethal at 29°C, 14 had a viability greater than 40 per cent at both temperatures, and the rest were semilethal at both temperatures.

The retests of the small sample of γ-ray- and MC-induced mutants suggest that these mutants may be quite stable. Of the γ-ray- and MC-induced mutants, all 8 and 9, respectively, remained ts upon retesting (Table 2). The apparent lability of the mutants induced by EMS as indicated by the retest data (only 90 confirmed out of 186) appears to be a characteristic of ts mutants induced by this mutagen. We have found repeatedly that some mutants classed as ts on the basis of one or two verifications appear to be ordinary lethals, semilethals, or subvitals in subsequent tests. Some of the variability may be attributable to gonadal mosaicism.[19] Accumulation of plus or minus modifiers for viability in the ts stock and the fairly extensive changes in the residual genome of the ts stock that occur when these stocks are outcrossed to the FM-6 balancer males must also be taken into account. Finally, there may exist genuine lability of the ts mutants themselves. At present, the experimental data are not adequate to distinguish between these alternatives. Therefore, the frequency of ts lethals recovered in Series B is taken as that given by the number of ts mutants confirmed by the retest, 59 ts in 919 lethals and semilethals (6.4%).

In Series B, however, the high rate of mutation induction will result in a number of chromosomes that carry more than one lethal mutation. If the probability of mutation induction on the X chromosome conforms to a Poisson distribution, then the frequency of chromosomes carrying a single mutation may be estimated. In Series B, it is estimated that 36.7 per cent of all chromosomes tested, or in other words, 60 per cent of all chromosomes scored as lethals, carry a single lethal mutation. In the remaining 40 per cent of the lethal chromosomes, two or more lethal mutations are expected. A ts mutant induced together with an ordinary lethal in a multiple mutant chromosome cannot be recovered by the screening procedures used.

In order to determine whether mutation induction does follow a Poisson distribution, 48 non-ts lethals induced by EMS in Series B were mapped genetically with the markers *y*, *cv*, *v*, *f*, and *car*.[20] Females heterozygous for the lethal and the markers were test-crossed and crossover values obtained in male and female offspring separately. Of the 48 lethals tested, 29 were expected to map as point mutants; the actual number was 31. These results indicate that lethal induction by EMS does conform to a Poisson distribution.

Preliminary genetic localization of 16 and 53 mutants in Series A and B, respectively, which were classified as ts in the retest, was carried out at 29°C. In Series

A, an average of 137 males was scored. Thirteen mutants mapped as single lethals or semilethals, 1 as an apparent multiple, and 2 yielded large numbers of wild-type males and could not be localized. In Series B, a smaller number of males was scored (average of 73 per ts) and 47 mutants mapped as single lethals, 1 as a multiple, 1 seemed to be an inversion, and 4 were not lethal when outcrossed to the marker stock. These data indicate that 60 out of 63 ts mutants that could be localized do map in a single region. Thus, the frequency of ts mutants among lethals induced by EMS in Series B is 10.7 per cent (no. ts mutants/no. single mutants).

In the maintenance of ts stocks we have observed that only 10 of the ts mutants recovered by screening at 17°C are either completely lethal or have very poor viability at room temperature (23°C). Use of 23°C as the permissive temperature shortens the time required for screening experiments without greatly reducing the yield of ts mutations.

Discussion.—The results reported indicate that a portion of lethals and semilethals induced by various agents is temperature-sensitive, the maximum number of ts mutants being induced by EMS. The arbitrary criteria used for classification of a mutant as a ts eliminate a large number of mutants which are subvital at 29°C but show appreciably more viability at 17°C, although still well below that of wild type. Such mutants comprise a considerable portion of induced and naturally occurring mutants as has been noted by other investigators.[11, 21, 22] Muller[23] found that mutation frequencies are significantly higher in cultures raised at 26.5°C than in cultures maintained 8°C cooler and attributed this difference to the induction of mutations by higher temperatures. It may be suggested that some of the excess recovered at the higher temperature represents spontaneously occurring ts lethal mutants which would be scored as nonlethals at the lower temperature.

Several indirect lines of evidence could be taken to indicate that a considerable proportion of the ts lethals detected are point mutants that may correspond to ts mutants studied in microorganisms:

(1) The mutagen referred to as ICR-100[15] is presumed to act as an acridine by inducing reading frame shift mutants.[24] None of the 200 sex-linked recessive lethals induced in *Drosophila* by this mutagen have been found to be ts.[24]

(2) γ-Rays and MC are known to cause extensive chromosome breakage and DNA degradation.[25–27] In our experiments, only 3.2 and 3.5 per cent, respectively, of the lethals induced by these mutagens are ts.

(3) In microorganisms, EMS induces a preponderance of missense mutations.[12] In *Drosophila*, we estimate that slightly more than 10 per cent of all EMS-induced lethals are ts.

(4) Most of the ts mutants induced in *Drosophila* can be readily mapped genetically in a single region (60 out of 63 mapped).

(5) Many of the ts lethals have a mutant phenotype at 17°C, an indication that even at 17°C the ts locus does not have wild-type activity.

Preliminary tests of the ts lethals recovered indicate that they can be used as tools for the kinds of genetic analysis that have been investigated primarily in microorganisms.

Summary.—Sex-linked recessive mutations that are lethals and semilethals at 29°C but survive at 17°C were recovered in *Drosophila melanogaster*. Of the lethals

and semilethals induced by γ-rays and mitomycin C, 3.2 and 3.5 per cent, respectively, are temperature-sensitive (ts), whereas it is estimated that at least 10.7 per cent of the lethals and semilethals induced by ethyl methanesulfonate are stable ts mutants. Such mutants provide a useful tool for a variety of genetic analyses.

The help of Dr. Harold Batho and Mr. Ken Yuen of the British Columbia Cancer Institute in irradiating the flies is gratefully acknowledged as is the discussion with Dr. Peter Larkin of this department on the statistical considerations.

* This research was supported by research grants AT(45-1)-1924 from the United States Atomic Energy Commission and A-1764 from the National Research Council of Canada.

[1] Campbell, A., *Virology*, **14**, 22–32 (1962).

[2] Epstein, R. H., A. Bolle, C. M. Steinberg, E. Kellenberger, E. Boy De La Tour, R. Chevalley, R. S. Edgar, M. Susman, G. H. Denhardt, and A. Lielausis, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 28 (1963), pp. 375–392.

[3] Wittmann, H. G., B. Wittmann-Liebold, and J. Jauregui-Adell, *Z. Naturforsch.*, **20b**, 1224–1234 (1965).

[4] Wittmann-Liebold, B., J. Jauregui-Adell, and H. G. Wittmann, *Z. Naturforsch*, **20b**, 1235–1249 (1965).

[5] Jockusch, H., *Biochem. Biophys. Res. Commun.*, **24**, 577–583 (1966).

[6] Horowitz, N. H., *Advan. Genet.*, **3**, 33–71 (1950).

[7] Nishihara, M., and W. R. Romig, *J. Bacteriol.*, **88**, 1220–1229 (1964).

[8] Edgar, R. S., and I. Lielausis, *Genetics*, **49**, 649–662 (1964).

[9] Langridge, J., *Australian J. Biol. Sci.*, **18**, 311–321 (1965).

[10] Igarashi, S., *Mutation Res.*, **3**, 13–24 (1966).

[11] Dobzhansky, T., and B. Spassky, *Genetics*, **29**, 270–290 (1944).

[12] Krieg, D. R., *Genetics*, **48**, 561–580 (1963).

[13] Fahmy, O. G., and M. J. Fahmy, *Genetics*, **46**, 1111–1123 (1961).

[14] Carlson, E. A., and I. I. Oster, *Genetics*, **47**, 561–576 (1962).

[15] Clarke, J. M., *Drosophila Information Service*, **37**, 139 (1963).

[16] Pelecanos, M., and T. Alderson, *Mutation Res.*, **1**, 173–181 (1964). We wish to thank Dr. E. B. Lewis for kindly providing us with his procedure for treating flies with EMS.

[17] Mukherjee, R., *Genetics*, **51**, 947–951 (1965).

[18] Ives, P. T., these PROCEEDINGS, **45**, 188–192 (1959).

[19] Epler, J. L., *Genetics*, **54**, 31–36 (1966).

[20] Bridges, C. B., and K. S. Brehme, *Carnegie Inst. Wash. Publ.*, **442**(1944).

[21] Spencer, W. P., and C. Stern, *Genetics*, **33**, 43–74 (1948).

[22] Tobari, I., *Genetics*, **54**, 783–791 (1966).

[23] Muller, H. J., *Genetics*, **13**, 279–357 (1928).

[24] Carlson, E. A., R. Sederoff, and M. Cogan, *Genetics*, **55**, 295–313 (1967). These workers used 24–28°C as the restrictive condition. We have found that most EMS-induced ts lethals survive at 23–24°C and die at 29°C. If ICR-100 does induce ts lethals which have the same critical temperature range, they might not be detectable by the conditions used by Carlson *et al.*

[25] Kaufmann, B. P., and H. Gay, *Repair from Radiation Damage*, ed. F. H. Sobels (New York: Pergamon Press, 1963), pp. 375–408.

[26] Weiss, J. J., *Progr. Nucleic Acid Res.*, **3**, 103–144 (1964).

[27] Shaw, M. W., and M. M. Cohen, *Genetics*, **51**, 181–190 (1965).