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Mutagenesis in *Caenorhabditis elegans*

I. A rapid eukaryotic mutagen test system using the reciprocal translocation, *eTI(III;V)*

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Summary

The advantages of developing mutagenicity tests using the nematode, *Caenorhabditis elegans*, are discussed and an efficient in vivo test for detecting heritable autosomal recessive lethals over 40 map units is described. The test uses the reciprocal translocation, *eTI(III;V)*, as a balancer. Dose–response curves for EMS (0.004–0.06 M) and γ-radiation (500–3000 R) were obtained. The spontaneous induction frequency for lethal mutations in 40 map units was found to be 0.06%. Mutations could be detected within 10 days and confirmed within another 5 days. From the point of view of *C. elegans* genetics, the EMS and γ-ray curves demonstrate that *eTI* can be used to test the efficacy of a particular mutagen in this organism. Although the present *eTI* protocol simultaneously screens hermaphrodite oocyte and sperm chromosomes, variations of the protocol that screen oocyte and sperm chromosomes separately are described.

Development of mutagenicity test systems using the small free-living hermaphroditic nematode, *Caenorhabditis elegans*, should prove extremely useful. Currently, in vivo tests for the detection of heritable mutations in gametes of complex eukaryotes are carried out most efficiently in *Drosophila melanogaster* (Würgler et al., 1977). The value of *C. elegans* as a test organism would lie in certain advantages it has over Drosophila, as well as in certain properties it shares with Drosophila. (For reviews see Brenner, 1974; Riddle, 1978; Herman and Horvitz, 1980.) In common with

Abbreviations: EMS, ethyl methanesulfonate; *LG*, linkage group; *eTI*, *eTI(III;V)*; *dpy*, dumpy; *unc*, uncoordinated; L4, larval stage 4; m.u., map unit.

Drosophila, *C. elegans* (1) has relatively few chromosomes (5AA, XX), (2) produces a relatively large number of progeny per parent (approx. 300 per wild-type hermaphrodite), (3) can mate and therefore transfer genes between individuals and (4) lays easily visible embryonic 'eggs' including aneuploids (Herman, 1978), whose survival frequency will be reduced by dominant lethal mutations and certain chromosomal rearrangements. The advantages of *C. elegans* over Drosophila are as follows. (1) It has a shorter generation time of 3.5 days at 20°. (2) Its common form of reproduction is as a self-fertilizing hermaphrodite. Thus, homozygosity of a new mutation can be achieved by the F2 generation and requires no brother-sister matings. (3) It has a relatively small size (1.5 mm long as an adult) so that at least 1000 individuals can survive in the space of a 15 × 100 mm petri plate. (4) It can feed either in liquid culture or on an agar surface. Thus comparing the effects of different concentrations of either water- or air-borne chemicals is relatively simple. Finally, (5) maintenance of strains is relatively easy because: (a) strains not in constant use can be frozen and stored indefinitely in liquid nitrogen; (b) larvae of an unfrozen strain can enter a dormant 'dauer larva' stage when the food supply is exhausted and survive until food is again presented; (c) strains can be maintained as hermaphrodites. The combination of the above properties has made possible an extensive analysis of the *C. elegans* genome within a relatively short period of time (Brenner, 1974; Herman and Horvitz, 1980). Thus the groundwork exists for developing mutagenicity tests using a variety of genetic end-points. The primary purpose of this report is to demonstrate the potential use of *C. elegans* as a test organism by presenting one type of test possible. The test should also prove useful for mutagenesis studies in *C. elegans* itself.

Our system measures the frequency of recessive lethal mutations induced within a sizable region of the *C. elegans* genome. This has been done previously for EMS-induced lethals on the X-chromosome (Brenner, 1974; Rose, 1979). The procedures used in those studies involved mating individual F1 hermaphrodites to males and screening for those F1's that produced less than 50% male cross-porogeny. Simpler systems are ones that screen for lethals over a large section of the genome balanced by a crossover suppressor (Herman, 1978). Such systems avoid both F1 matings and male-scoring. We have recently shown that the homozygous viable reciprocal translocation, *eT1*(III;V), can be used to balance the right half of *LGIII* and the left half of *LGV*. Together these account for a total of 39 map units and constitute the largest region as yet balanced in *C. elegans* (Rosenbluth and Baillie, 1981). In our previous report we demonstrated that a screening procedure with *eT1* can detect lethal mutations in the two balanced regions. We have now obtained dose–response curves for the known mutagens, EMS and γ-radiation, in order to determine what dosages can be detected efficiently using this '*eT1* system'.

Our data are also valuable for the genetics of *C. elegans* itself. Due to the lack of any other convenient system, no spontaneous induction rate has been published for this organism and the only mutagen dose–response curve reported so far is that of Rose (1979) for EMS-induced sex-linked lethals. Our report demonstrates that a spontaneous induction rate and dose–response curves for autosomal lethals can be obtained relatively easily using *eT1*. The induction frequencies measured with the

present protocol are those in both hermaphrodite gametes (oocytes and sperm) combined. However, modifications of the protocol are described which would allow the frequencies in oocytes and sperm to be measured separately.

Materials and methods

General

The nomenclature follows the uniform system adopted for *Caenorhabditis elegans* (Horvitz et al., 1979). Nematodes were cultured in petri dishes on a simple agar nematode growth medium streaked with *Escherichia coli OP50*. For details of this as well as procedures for observing and handling the worms see Brenner (1974). The following homozygous *C. elegans* (var. Bristol) strains were obtained from the MRC, Cambridge, England: N2 (wild-type); CB364, *dpy-18(e364)III;* CB 177, *unc-46(e177)V*; CB873, *eT1(III;V)*. A partial genetic map for *C. elegans* is shown in Fig. 1.

The test strain

Hermaphrodites from a homozygous *dpy-18;unc-46* strain were mated to *eT1 / +* males. Phenotypically wild-type hermaphrodite offspring were progeny tested and a heterozygous test strain, *dpy-18;unc-46 / eT1* was started from one of them. The characteristics of this strain and of *eT1* have been described previously (Rosenbluth and Baillie, 1981). Briefly: *eT1* is a reciprocal translocation between linkage groups *III* and *V* and is associated with a recessive *unc* mutation which fails to complement *unc-36(e251)III*. As a homozygote *eT1* is viable and its Unc-36 phenotype is easily identified. In worms heterozygous for *eT1*, crossing over is completely suppressed between *sma-2* and *unc-64* on *LGIII* and between *unc-60* and *dpy-11* on *LGV*. Furthermore, the segregation patterns from such heterozygotes show that only euploid zygotes develop to adulthood. Thus the only self progeny from the test-strain heterozygotes are: homozygous *dpy-18;unc-46* (phenotypically Dpy-18 Unc-46); the parental heterozygote (phenotypically wild-type); and homozygous *eT1* (phenotypically Unc-36), in the ratio of 1 : 4 : 1. Even though only 36% of its eggs survive, the

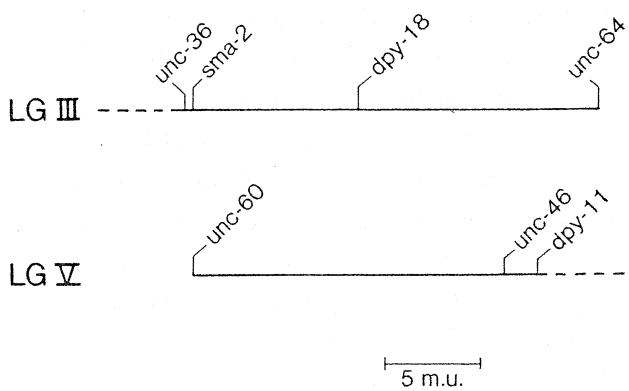


Fig. 1. Regions balanced by *eT1(III;V)*. Right half of *LGIII* and left half of *LGV*.

heterozygote continues to constitute the largest progeny class in subsequent generations. Because of this and because it develops slightly faster than either of the two homozygotes, the strain remains stable without the necessity of selection. If a recessive lethal mutation is induced in one of the normal homologues anywhere within the balanced regions, there will be only wild-type and Unc-36 progeny (i.e. no Dpy Uncs).

Mutagenesis treatments

A population of test-strain worms was rinsed off a stock plate into a 15-ml centrifuge tube and washed 3 times with M9 buffer (Brenner, 1974). The worms were then treated with one of the mutagens.

(1) EMS treatment. Washed worms were incubated in a 12 × 75 mm round-bottomed polystyrene tube at 20° for 4 h in 4 ml M9 buffer containing a known concentration of EMS (Sigma cat. No. M-0880). Using a pasteur pipette the worms were then spotted onto a culture plate. After 1 h young gravid phenotypically wild-type hermaphrodites (Po's) were transferred to a fresh culture plate and left there for another hour before use. The purpose of this step was to allow the in utero embryos proesent during mutagenesis to be expelled, and thus be removed from the F1 sample.

(2) γ-Radiation. Washed worms were spotted onto a culture plate and young gravid wild-type Po's were transferred onto a fresh plate on which they were irradiated. Radiation was carried out using a ^{60}Co radiation unit (Gammacell 200, Atomic Energy of Canada Ltd.). The dose rate varied from 296 R/min to 263 R/min. Immediately after irradiation, the worms were transferred first to one fresh plate for 1 h then to another one where they were again left for 1 h before further use.

Screening for recessive lethals in the balanced regions of the normal homologues

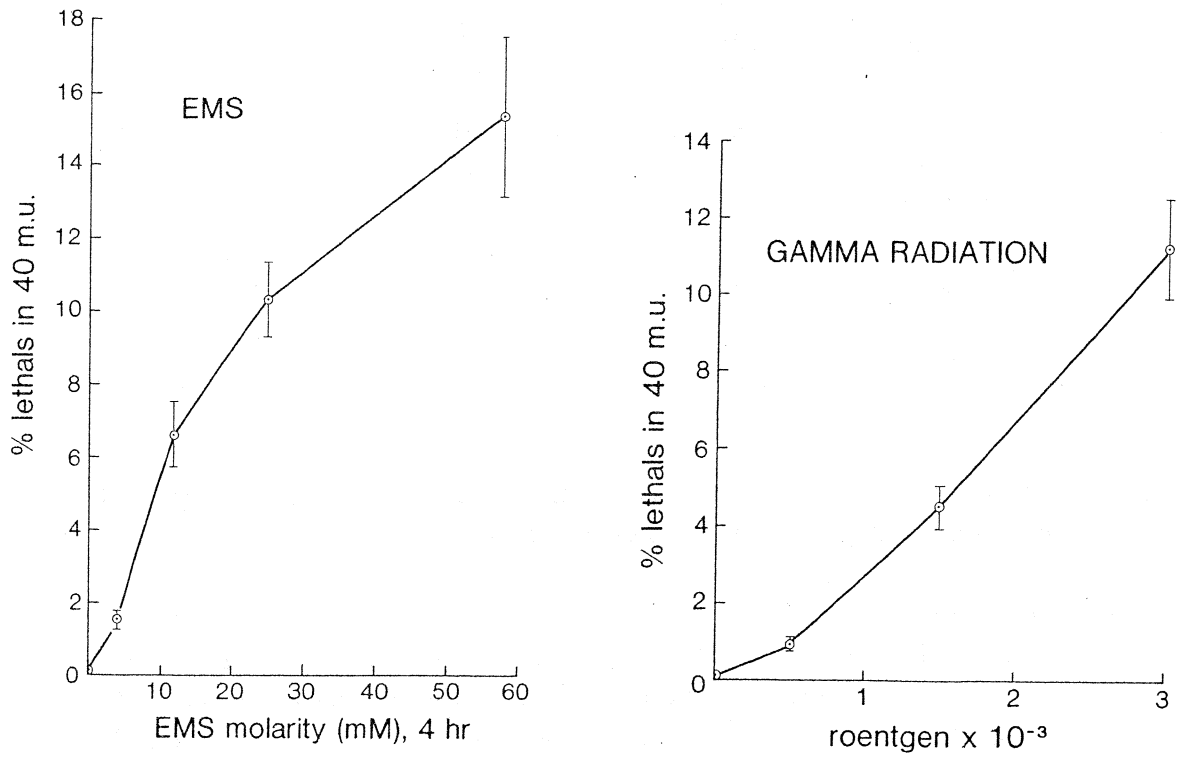
Single treated Po hermaphrodites were placed on individual culture plates, transferred to fresh plates after 24 h, and removed after another 24 h. The plates were kept at 20°. Starting on the 4th day, single F1 heterozygotes were placed on individual plates (either 10 × 35 mm or 20 × 60 mm). To insure that no Po was used whose genotype already carried a spontaneous lethal mutation in the screening region, the F1 progeny of each Po was checked for the presence of Dpy Unc worms. From any one Po hermaphrodite all F1 heterozygotes were used. A heterozygous F1, produced from a Po oocyte or sperm carrying a lethal within the screening region, will produce no Dpy Unc F2's. Therefore, when ready (approximately 4 days later), the F2 progeny on each plate was screened for *adult* Dpy Unc worms. 'Adulthood' was established by the detection of internal eggs. From every plate showing no such Dpy Uncs, a putative lethal heterozygous strain was kept. A strain was confirmed as carrying a lethal mutation inside one of the crossover suppressed regions (or within one map unit of them) if no adult Dpy Uncs appeared among at least 250 offspring from heterozygous hermaphrodites. In the absence of any linked lethals the frequency of Dpy Uncs would be 1/6. In the presence of a linked lethal on *LGIII* or *LGV*, in the region *not* balanced by *eT1*, the frequency of Dpy Uncs would be less than 1/6.

A lethal 1 m.u. from the balanced region would produce Dpy Uncs with a frequency of 1/250. At low mutagen doses, 4–5 heterozygotes of a putative lethal strain usually gave enough progeny to confirm the lethality. At higher doses, brood sizes were often reduced so that more heterozygotes had to be used. In addition, if a Dpy Unc was detected on a stock plate of a particular strain, the strain was discarded. Thus the criterion for confirming lethality within the defined region was extremely rigorous for strains with normal fecundity. Strains that produced adult-size Dpy Uncs, all of which lacked eggs, were confirmed by isolating Dpy Uncs as L4's and checking for the absence of both internal and expelled eggs. These 'adult-egg-steriles' were included in the lethal score.

Results

Stability of the test strain

After its construction, the hermaphrodite test strain, *dpy-18;unc-46/eT1*, was maintained unfrozen on agar medium for several months, then stored in liquid nitrogen for 6 months and subsequently again maintained unfrozen for a year. While it was unfrozen, several generations were frequently allowed to elapse without reselecting heterozygotes. In spite of this, the strain never broke down — i.e. both the translocation chromosomes and the marked homologues were maintained. On two occasions we detected the presence of a spontaneous lethal mutation inside the



Figs. 2 and 3. Dose–response curves for the induction of recessive lethals in the regions balanced by *eT1(III;V)*. Bars indicate ± standard error. 2, EMS. 3, γ-radiation.

TABLE 1

INDUCTION OF RECESSIVE LETHALS BY VARYING DOSES OF EMS AND γ-RADIATION

Dose	Date of Expt.	Number of F1's tested	Number of lethals	Lethals (%)
Control				
	21/06/81	1812	2	0.11
	27/09/82	1386	0	0
	Total	3198	2	0.06
EMS				
0.004 M	03/06/81	959	12	1.3
	11/01/82	788	14	1.8
	Total	1747	26	1.5
0.012 M	04/05/81	348	23	6.6
	13/11/81	483	32	6.6
	Total	831	55	6.6
0.025 M	03/06/81	363	40	11.0
	11/01/82	479	47	9.8
	Total	842	87	10.3
0.058 M	04/05/81	261	40	15.3
γ-Radiation				
500 R	27/07/81	1084	9	0.83
	22/10/81	360	4	1.1
	30/10/81	682	5	0.73
	05/07/82	875	9	1.0
	05/07/82	913	7	0.75
	05/07/82	875	12	1.4
	Total	4789	46	0.96
1500 R	26/06/81	364	22	6.0
	07/12/81	485	17	3.5
	29/03/82	787	35	4.4
	Total	1636	74	4.5
3000 R	26/06/81	188	23	12.2
	29/03/82	439	47	10.7
	Total	627	70	11.2

crossover suppressed region. Therefore, when the strain was 'in-use', a periodic check for the presence of all three phenotypes was necessary.

The dose–response data

The dose–response data are given in Table 1 and Figs. 2 and 3. In the case of EMS the 'dose' refers to the concentration of EMS that the worms were exposed to for 4 h at 20°. The 'response' is expressed as the percentage of fertile F1's that carried at least one lethal mutation within the 40 map units screened. (F1's that were sterile or whose strains continually gave extremely low fecundity were not used).

Since the F1's were heterozygotes, this equals the frequency with which at least one lethal mutation was induced in the 40 m.u.

Table 1 lists the individual experiments separately and shows that reproducibility was good for both mutagens. The lethal frequencies given in Figs. 2 and 3 are those for the total F1's at each dose. Frequencies higher than those shown could not be studied easily since they were accompanied by too much sterility and too many visible mutations that confused the screening process. To determine whether or not the curves are linear at low dosages will require more data. (See the Discussion for comments on the apparent non-linearity of the γ-ray curve.)

Sensitivity of the eT1 system as a mutagenicity test

The sensitivity of the system in terms of the lowest dose it can detect conveniently, depends on two factors: the number of treated F1 individuals that can be tested conveniently, and the spontaneous mutation rate of the system. We found that for doses giving mutation rates of 1–2% or lower, 2000 F1's could be tested within 2–2.5 weeks by two people. If we use an average spontaneous mutation rate of 0.15% (95% confidence limits of the data are 0.01–0.22%), then inspection of a table published by Würgler et al. (1977) shows that, with a sample size of 2000, a mutation rate of about 0.65% can be detected as being significantly larger than the spontaneous rate. This means that mutagen doses corresponding to about 400 R (Fig. 3) can be detected and confirmed within 2.5 weeks.

Discussion

The primary aim of this study was to show that *C. elegans* can be used as a mutagen test organism. To this end we have demonstrated the use of an in vivo test for autosomal recessive lethals and obtained dose–response curves for two known mutagens: EMS and γ-radiation. The fact that *C. elegans* survives in liquid medium, made consistent treatment with a water-soluble chemical, like EMS, a relatively simple procedure. Although more data are needed to determine their exact shapes, our curves show that with the present protocol the *eT1* test detects dose differences within the ranges of 0.004–0.06 M EMS and 500–3000 R γ-radiation.

For the purpose of testing unknown agents, our test compares favorably with those for recessive lethality in Drosophila, both with respect to speed and convenience. Foremost is the time element. We estimate that the effects of mutagen doses comparable to 400 R γ-radiation could be detected within 10 days and confirmed within another 5 days. In Drosophila such tests would require at least 4 weeks for sex-linked lethals and 6 weeks for autosomal ones. It may be possible to increase the efficiency of the present *eT1* protocol by changing the criterion for lethality from 'absence of any *egg-bearing* adult Dpy Uncs' to 'the absence of any *adult-size* Dpy Uncs'. About 10% of each lethal sample consists of 'adult egg-steriles' (mutations that permit development to adulthood but prevent egg formation). Since the confirmation of these is tedious, the change in criterion would simplify the retests (and decrease the number of detected lethals by only 10%). In terms of convenience,

the *eT1* test has several favorable aspects. In the first place, maintenance of the heterozygous test strain is extremely easy even in the unfrozen state, since selection at every generation is not necessary. Secondly, the protocol used in this study requires no matings. Thirdly, since the tested chromosomes come from both treated oocytes and treated sperm, a mutagen acting in only one of these gametes does not escape detection. Finally, a large number of the confirmed heterozygous lethal strains can be accumulated for further analysis without causing a stockkeeping burden, since they can be stored in liquid nitrogen. At a later date the mutations can be outcrossed (segregated away from *eT1*) and mapped to specific sites within the screened region (Rosenbluth and Baillie, 1981). They can then be analysed for association with deletions or other chromosomal aberrations.

The convenience with which lethal mutations are detected using the *eT1* system should also be extremely useful for the genetics of *C. elegans* itself. It provides methods for determining factors affecting mutagenesis in *C. elegans* and for testing the efficacy of a particular mutagen in this organism. The dose–response curves reported here demonstrate the latter and can serve as guidelines for choosing appropriate EMS and γ-ray doses. Our EMS curve for autosomal lethals in 40 m.u. compares remarkably well with that for sex-linked lethals obtained by Rose (1979). It is important to note, however, that in both these studies the treated Po hermaphrodites produced their progeny by self-fertilization, so that a lethal mutation carried by an F1 could have been induced in either an oocyte or a sperm. Consequently each lethal frequency shown in the curves represents an average of the combined induction frequencies occurring in oocytes and sperm. Since mutagens may have different effects in the two types of gametes, the curves are applicable to only those cases where F1's are the self-progeny of treated Po's. There are, however, experimental designs in which F1's are the progeny of treated Po's (hermaphrodites or males) crossed to untreated partners. For such cases it would be desirable to determine lethal frequencies in oocytes and sperm separately. This can also be done using *eT1*. By crossing treated *dpy-18;unc-46/eT1* males * with untreated homozygous *eT1* hermaphrodites, in the Po generation, the induction frequency of lethals in *male* sperm will be measured. Alternatively, crossing untreated *dpy-18;unc-46/eT1* males with treated homozygous *dpy-18;unc-46* hermaphrodites will give the frequency in only oocytes. The frequency in *hermaphroditic* sperm will be the difference between the oocyte frequency and twice that obtained from self-fertilizing hermaphrodites. Thus separate curves for each gamete may be obtained.

Two further aspects of our study are relevant to *C. elegans* genetics. First is the shape of the γ-ray curve which surprised us. Although the data are still sparse there appears to be a lag at low dosage. Preliminary results by others in our laboratory (Pilgrim and Rogalski, unpublished results) had suggested that oocytes are far less sensitive to this type of mutagenesis than are sperm. We had, therefore, expected that our γ-ray curve (for self-fertilizing Po's) would reflect predominantly induction in hermaphroditic sperm and would be linear, as are similar curves for Drosophila males (for a review see Sankaranarayanan and Sobels, 1976). The presence of a lag is

* Males homozygous for either *dpy-18;unc-46* or *eT1* cannot mate.

not surprising if the rates in oocytes are actually greater than we had assumed and contribute substantially to the shape of our curve, since such curves for Drosophila oocytes are also non-linear at low dosage (Sankaranarayanan and Sobels, 1976). On th other hand, if this is not the case, then the lag may indicate that γ-ray mutagenesis occurring in the sperm of *C. elegans* is different from that in Drosophila sperm. Further experiments, including the testing of *C. elegans* oocyte and sperm sensitivities separately (as outlined above), are needed to clarify this point.

The spontaneous lethal rate is also of interest. It is the first such rate to be reported for *C. elegans*. Assuming that the frequency of 0.06% per 40 m.u. reflects the average spontaneous rate in the genome as a whole, and that strain differences are relatively small, then a new spontaneous lethal will be carried in 1 out of every 119 self-fertilizing hermaphrodites, each generation (taking 40 m.u. to be about 1/7th of the genome). These mutations will, of course, be lost quickly in strains kept unfrozen and without balancers. However, most *C. elegans* strains are kept in liquid nitrogen for long periods of time. The fact that our test strain can also be kept frozen will permit us to determine whether spontaneous lethal mutations accumulate with time in such non-proliferating strains.

In conclusion, we have shown that the *C. elegans eT1* system provides a relatively fast and convenient in vivo eukaryotic mutagenicity test and is a useful tool for *C. elegans* genetics. The genetic end-point of this system is recessive lethality over a large portion of the genome. In Drosophila such tests are considered the most useful ones for mutagenicity testing (see Vogel and Sobels, 1976). However, as in Drosophila, screens for other end-points such as, non-disjunction of the X-chromosome, dominant lethality, reversions and translocations are also possible in *C. elegans*. Mutagenicity testing procedures using these indicators could, therefore, be developed. Thus we believe that the advantageous properties of *C. elegans* such as, generation time, mode of reproduction, size of animal, methods of culturing and stockkeeping, will make this a valuable eukaryote for in vivo mutagenicity testing.

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