

## Reduction of Background Mutations by Low-Dose X Irradiation of *Drosophila* Spermatocytes at a Low Dose Rate

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A sex-linked recessive lethal mutation assay was performed in *Drosophila melanogaster* using immature spermatocytes and spermatogonia irradiated with X rays at a high or low dose rate. The mutation frequency in the sperm irradiated with a low dose at a low dose rate was significantly lower than that in the sham-irradiated group, whereas irradiation with a high dose resulted in a significant increase in the mutation frequency. It was obvious that the dose–response relationship was not linear, but rather was U-shaped. When mutant germ cells defective in DNA excision repair were used instead of wild-type cells, low-dose irradiation at a low dose rate did not reduce the mutation frequency. These observations suggest that error-free DNA repair functions were activated by low dose of low-dose-rate radiation and that this repaired spontaneous DNA damage rather than the X-ray-induced damage, thus producing a practical threshold. © 2007 by Radiation Research Society

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### INTRODUCTION

The dose–response relationship between ionizing radiation and induced mutation frequency was reported as early as 1930 using a sex-linked recessive lethal assay in mature sperm of the fruit fly *Drosophila melanogaster*. Oliver (1) showed that between 285 r (2.7 Gy) and 4560 r (43.5 Gy), the X-ray dose and mutation frequency had a linear relationship without any threshold. Most succeeding studies supported the linear relationship (2). Studies using bacteria, yeast, cultured mammalian cells and mice also showed a linear dose response (3). Since then, the so-called linear non-threshold (LNT) model has been widely accepted as a

basis for estimation of radiation risks to humans (4). At that time, the LNT model looked quite reasonable; cancer risk is proportional to mutation rate, and the mutation rate is proportional to radiation dose, and cancer risk is therefore proportional to radiation dose.

In 1982, Russell and Kelly (5) demonstrated that mutation frequency in murine spermatogonia is dependent not only on the total radiation dose but also on the dose rate. The same cumulative doses resulted in different mutation frequencies when the dose rates were different; this is called the dose-rate effect. Generally, an acute irradiation is more damaging than chronic irradiation. It was inferred that in acutely irradiated cells, radiation-induced DNA damage is not repaired completely, because the repair capacity is limited, leading to mutations. It was shown that DNA repair is a key function in determining radiosensitivity in *Drosophila* (6, 7) and in cultured human cells (8). The DNA repair function is also involved in the induction of mutation (9) and possibly in the formation of thresholds. In Oliver's experiment (1) and in most succeeding studies (2), only mature sperm were used for the lethal mutation assay. Mature sperm have no cytoplasm and no repair activity (10, 11). We reported previously (12) that in the somatic mutation assay in *Drosophila* there is a threshold around 1 Gy and that a mutation in DNA repair function decreases the threshold value. It is possible that even in germline mutation assays, a threshold can be detected when repair-proficient immature sperm are used. Here we report the existence of a threshold in the sex-linked recessive lethal assay using immature sperm. The involvement of the DNA repair function in establishing a threshold is indicated.

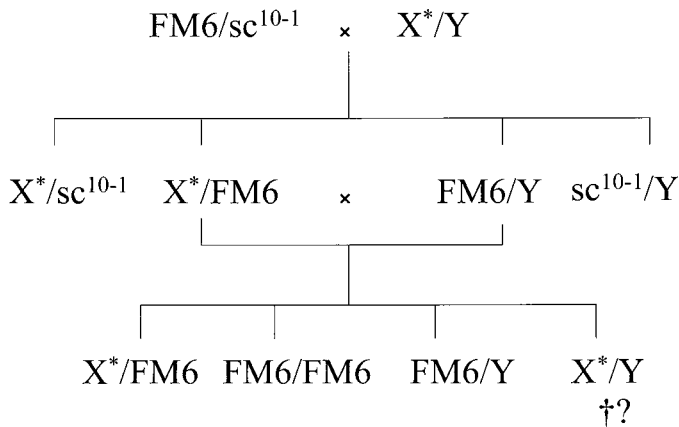
### MATERIALS AND METHODS

#### *Fly Strains and Culture Condition*

Canton-S was used as a wild-type strain. For a mutant strain defective in the excision repair function, *y mei-9<sup>v</sup> f y<sup>+</sup>* was used. As an X chromosome balancer, *FM6* was used [for details of the wild-type, mutant genes and balancer, see ref. (13) and FlyBase (14), a database of the *Drosophila* genome]. Flies were fed with conventional fly culture medium [8% (w/v) cornmeal, 8% (w/v) glucose, 8% (w/v) dry yeast and 0.8%

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**FIG. 1.** Mating scheme for the sex-linked recessive lethal test. Each irradiated or sham-irradiated male was mated to an *FM6/sc*<sup>10-1</sup> virgin female in separate vials. F<sub>1</sub> females carrying the *FM6* chromosome were collected, and single-pair matings with *FM6/Y* male were made. Only one *FM6/X\** female was used from each parental mating. Another 15 days later, F<sub>2</sub> flies were examined, and vials from which no wild-type males emerged were judged as candidates for sex-linked recessive lethals. The total numbers of X chromosomes examined were 2623 (10 Gy, 0.5 Gy/min), 3694 (0.2 Gy, 0.5 Gy/min), 3050 (10 Gy, 0.05 Gy/min), 3036 (0.2 Gy, 0.05 Gy/min) and 11,762 (total of all four sham-irradiated groups).

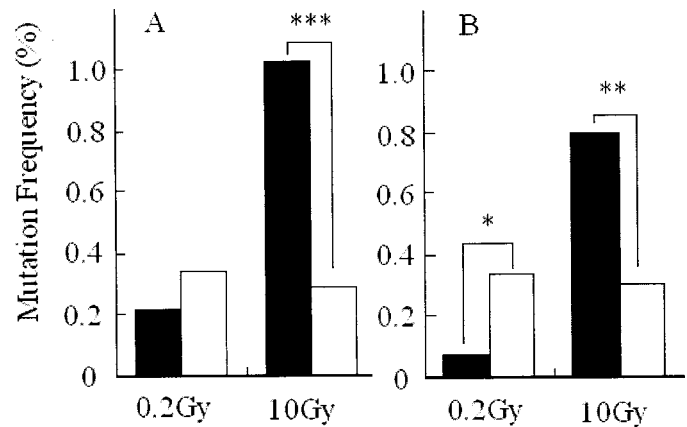
(w/v) agar in tap water with 0.8% (v/v) propionic acid as a mold inhibitor] and were reared in a constant temperature room at 24 ± 0.5°C with a 12-h light-dark cycle. Flies were allowed to lay eggs for 4 h, and then parents were removed. Eggs were incubated at 24°C for 4 days to hatch into larvae before they were irradiated.

#### X Irradiation

The third instar larvae were irradiated with X rays at 96 ± 2 h after egg laying. Sperm at this developmental stage are a mixture of spermatocytes, spermatogonia and germline stem cells (15). Almost all the sperm are pre-meiotic and are assumed to have active DNA repair functions (6, 16). Irradiation was done with an X-ray generator (MBR-1505R2, Hitachi, Tokyo) operated at 150 kVp, 2 or 5 mA with 1 mm aluminum + 0.2 mm copper filters. The upper surface of the fly culture medium where larvae were crawling was placed 23 or 53 cm from the X-ray source. The dose rate was 0.5 or 0.05 Gy/min depending on the distance and tube current. The temperature of the room in which the X irradiation was performed was also set at 24°C to avoid any artifact from a temperature change during the experiment. After irradiation, flies were brought back to the culture room and were reared under normal conditions until their eclosion.

#### Sex-Linked Recessive Lethal Test

The mating scheme is shown in Fig. 1. At the fifteenth day of egg laying, adult flies that had eclosed from pupal cases were anesthetized with carbon dioxide gas, and males were collected. Using these males, around 400 sets of single-pair mating with an *sc*<sup>10-1</sup>/*FM6* virgin female were made for one experiment (200 irradiated and 200 sham-irradiated males). Parental flies were removed 7 days after crossing. At the fifteenth day of crossing, F<sub>1</sub> females carrying an *FM6* chromosome were collected, and 400 sets of single-pair mating with an *FM6/Y* male were made. To avoid the possibility that the single pre-meiotic mutation would be amplified by cell division and isolated several times, only one *FM6/+* female was used from each parental mating. Another 15 days later, F<sub>2</sub> flies were examined, and vials from which no wild-type males emerged were kept as candidates for sex-linked recessive lethals. They were checked



**FIG. 2.** Frequencies of sex-linked recessive lethal mutations. Solid bars represent mutation frequencies in groups irradiated with the indicated doses. The dose rate was 0.5 Gy/min (panel A) or 0.05 Gy/min (panel B). Open bars represent the corresponding sham groups. For irradiation with 0.5 Gy/min (panel A), the mutation frequency in the 10-Gy group was significantly higher than that in the corresponding sham group ( $P < 0.001$ ). The mutation frequency was reduced in the 0.2-Gy group, but this difference was not statistically significant. For irradiation with 0.05 Gy/min (panel B), the reduction in the 0.2-Gy group became significant ( $P < 0.05$ ). The mutation frequency in the 10-Gy group was still significantly higher ( $P < 0.01$ ).

for carrying an X-ray-induced sex-linked lethal mutation in the next generation. Thirteen to 18 repeat experiments were done for each dose/dose-rate condition. The statistical significance of the difference in the lethal mutation frequencies in the irradiated and the corresponding sham-irradiated groups was confirmed by  $\chi^2$  analysis.

#### Genetic Mapping

Established sex-linked recessive lethals were analyzed for their genetic loci using a mapping strain carrying sex-linked external marker genes. Virgin females heterozygous for a lethal were crossed to *y sc cv v f y*<sup>+</sup> males (13, 14). Recombinant F<sub>2</sub> males were analyzed for expression of marker genes, and the locus of the lethal mutation was identified according to the classical mapping strategy.

## RESULTS

#### Dose-Response Relationship of Wild-Type Flies at a High Dose Rate

When the third instar larvae of wild-type flies were irradiated with 10 Gy at a high dose rate (0.5 Gy/min), an increase in the sex-linked recessive lethal mutation frequency compared with that in the sham-irradiated group was observed (Fig. 2). Nonlethal mutations in external morphology were found occasionally, but they were not used for the calculation of mutation frequencies. The lethal mutation frequency in the sham-exposed group was 0.29% (8 lethals/2736 X chromosomes), which is similar to the data for sham-exposed groups in previous reports (17–19). The mutation frequency at 10 Gy was 1.03% (27/2623), which was significantly higher than that in the corresponding sham-irradiated group ( $P < 0.001$ ). Although the mutation frequency in irradiated groups will differ according to the experimental conditions (temperature, developmental stage

of flies at irradiation, dose rate, X-ray tube voltage or filters), our data (1.03% at 10 Gy) fitted the linear regression equation proposed by Oftedal (17). The mutation frequency at 0.2 Gy was 0.22% (8/3694), which was slightly lower than that in the sham-irradiated group (11/3228 = 0.34%), though the difference was not statistically significant. The dose–response relationship at a high dose rate was compatible with the LNT model.

#### *Dose–Response Relationship of Wild-Type Flies at a Low Dose Rate*

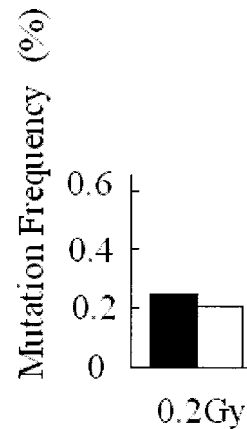
When wild-type flies were X-irradiated at a low dose rate (0.05 Gy/min), the dose–response relationship was found to be quite different from that predicted by the LNT model (Fig. 2). The mutation frequency at 10 Gy was 0.79% (24/3050), which was lower than that in the high-dose-rate experiment, but still significantly higher ( $P < 0.01$ ) than that in the sham-irradiated group (9/2981 = 0.30%). However, the mutation frequency at 0.2 Gy was 0.07% (2/3036), which was significantly lower than that in the sham-irradiated group (9/2767 = 0.33%,  $P < 0.05$ ). This result is not consistent with the LNT model. A J- or U-shaped dose–response relationship was evident. There is a threshold between 0.2 and 10 Gy below which no increase in mutation frequency is detected.

#### *Loci of Lethal Mutations*

The genetic loci of isolated lethal mutations (46 X-ray-induced and 11 spontaneous) were identified. Most of these were mapped to single loci on the X chromosome. In the X-irradiated groups, 26 of the mutations (among 43 mapped lines) were between *f* and the centromere, the same region as the salivary gland chromosome bands 16 to 20. Six of the mutations were between *y sc* and *cv*, one between *cv* and *v*, and 10 between *v* and *f*. This was comparable to the previous report that two-thirds of lethals were located in chromosome band 20 (2). However, four mutants (three of the 46 X-ray-induced and one of the 11 spontaneous) could not be mapped precisely. Since our classical genetic mapping strategy depended on recombination between homologous X chromosomes, it suggested that chromosomal recombination was suppressed, possibly by a large deletion, inversion or translocation.

#### *Dose–Response Relationship of DNA Repair-Defective Flies*

A repair-defective mutant strain was used instead of the wild-type strain to confirm the involvement of repair function in causing the threshold. Male flies carrying the *mei-9<sup>a</sup>* mutation (20) and therefore defective in the error-free nucleotide excision repair function were X-irradiated with 0.2 Gy at a low dose rate (0.05 Gy/min). The irradiation was carried out at the same developmental stage used in the experiments with the wild-type flies (96 ± 2 h). The mutation frequency was 0.25% (8/3243) in the irradiated



**FIG. 3.** Sex-linked recessive lethal mutation frequencies in the excision repair-defective *mei-9<sup>a</sup>* flies. The solid bar represents the irradiated group; the open bar represents the control group. A low-dose (0.2 Gy), low-dose-rate (0.05 Gy/min) irradiation did not cause a reduction in mutation frequency.

group and 0.21% (7/3312) in the sham-irradiated group. These two values are quite similar (Fig. 3), and it was shown that *mei-9<sup>a</sup>* mutant flies did not exhibit a J-shaped dose–response relationship and that the defects in the error-free repair function extinguished the threshold.

## DISCUSSION

It was found that the dose–response relationship between X irradiation and mutation frequency was not linear when repair-proficient immature larval sperm were irradiated at a low dose rate. Recently, U- or J-shaped dose responses have been recognized as being the rule in toxicology and in radiobiology rather than the rare exceptions (21, 22). We must remember that the LNT is a model that is used to avoid an underestimation of the radiation effects in formulating protection criteria. The actual dose–response relationship is not always linear, especially in the low-dose region or at low dose rates.

Since a linear dose–response relationship has been well established when mature sperm are irradiated (2), and since adult testes include sperm in various developmental stages, it is interesting to examine the immature adult sperm to see whether the dose–response relationship is also J-shaped. It is possible, if not probable, that immature adult sperm respond in a different manner from larval immature sperm. Savina *et al.* (23) found that an adaptive response to alkylating agents occurred only when larvae were primed. When immature spermatogonia of adults were primed, an adaptive response was not seen. However, when adult flies were irradiated with a high dose of X rays, a lower induced mutation frequency was observed in spermatogonia compared with more mature sperm (24, 25). This strongly suggests that DNA repair function is activated by radiation in immature adult sperm. The difference in response to chemicals between larval and adult spermatogonia may result from differences in metabolism between larvae and adults.

It is important to identify the mechanisms that result in the non-linear dose–response relationship. *Mei-9<sup>a</sup>* mutant flies did not exhibit a threshold, suggesting that the nucleotide excision repair function is involved in threshold formation. Although excision repair is not effective in repairing double-strand breaks induced by radiation (26), it should effectively repair damage such as pyrimidine dimers, base damage or adducts. These kinds of damage are either induced by X radiation through radical reaction or caused spontaneously. In the sex-linked recessive lethal assay, recessive lethal genes on the X chromosome that are heterozygous in the gene pool of the test population are not transmitted to the sperm of the tester males and therefore are not detected as spontaneous lethals in the control or the treated group. All spontaneous mutations resulted from errors in the repair of spontaneous DNA damage caused during spermatogenesis of the tester males. It is inferred that 0.2 Gy radiation activated the error-free nucleotide excision repair system (which is inactive or at baseline activity if the flies are not irradiated), decreasing the spontaneous mutation frequency, compensating for an increase in the radiation-induced mutation, or even exceeding it to reduce the total mutation frequency. This should produce a practical threshold.

The activation of an inducible repair system is generally recognized as the basic mechanism of the adaptive response. Therefore, it is possible to say that we have observed a cellular adaptive response by not applying a challenging dose. However, it is unique that 0.2 Gy given at 0.05 Gy/min induced an adaptive response while the same dose given at 0.5 Gy/min did not. It is well known that the same dose given at different dose rates results in different frequencies of induced mutations. The higher the dose rate, the higher the mutation frequency. The reduction in the spontaneous mutation rate, on the other hand, should be independent of the dose rate. Thus the resulting (X-ray-induced + reduced spontaneous) mutation frequency should be different at different dose rates. Wang *et al.* (27) found a clear dose-rate dependence of the radioadaptive response in mice. Their end point was teratogenesis, and they presented a complicated discussion on the mechanism of the dose-rate dependence. Involvement of the cell cycle, growth factors and so on was hypothesized. However, in our case, simple addition of spontaneous and X-ray-induced mutations can explain the apparent dose-rate dependence.

Excision repair may not be the only factor that caused reduction in mutation frequency in flies irradiated with a low dose at a low dose rate. Photo-reactivation, another error-free repair function, is known to occur in *Drosophila* (28). Activation of this function may also influence the spontaneous mutation frequency. Apoptosis may also play a role in the formation of the threshold. Involvement of apoptosis in threshold formation has been observed in embryonic development in mice (29). In *Drosophila*, germinal selection was observed (30). The term “germinal selection” means elimination of sperm with DNA damage, and

therefore, it is not necessarily equal to the apoptosis itself but quite probably is related to it. However, the *mei-9* mutant did not show a J-shaped dose response, which indicates that excision repair function is one of the important elements in the threshold formation.

The spontaneous mutation frequency in nucleotide excision repair-defective mutant flies was slightly lower than that in the wild-type flies, though the difference was not significant. It was reported (31) that the spontaneous mutation frequency in the *mei-9* mutant accumulated through 50 generations was several times higher than in the wild type, which seems contradictory to our results. However, in the sex-linked recessive lethal assay, accumulated lethal mutations are not detected, and all of the lethal mutations detected were induced during spermatogenesis of the tester males. Therefore, it is possible that a difference between mutant and wild type was not observed. It is known that XPF proteins in CHO cells are not incorporated into the active nucleotide excision repair (NER) protein complex without DNA damage (32). Since *mei-9* is a homologue of *Xpf*, it is possible that *Drosophila* Mei-9 protein also is not part of the NER complex in nonirradiated flies. If so, mutation in the *mei-9* gene would not influence the background mutation frequency. The *mei-9* mutation produces an effect only in the irradiated flies. Hsia *et al.* (33) showed that apoptosis was increased in testes of the excision repair-deficient *ERCC1* mice. It is possible that apoptosis is activated in the spermatocytes and spermatogonia of *mei-9<sup>a</sup>* flies in our experiment and thus that germ cells with spontaneous DNA damage were abolished during spermatogenesis, resulting in a reduction of spontaneous mutation.

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