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RBE-LET Relationships of High-LET Radiations in *Drosophila* Mutations

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The relative biological effectiveness (RBE) of ^{252}Cf neutrons and synchrotron-generated high-energy charged particles for mutation induction was evaluated as a function of linear energy transfer (LET), using the loss of heterozygosity for wing-hair mutations and the reversion of the mutant *white-ivory* eye-color in *Drosophila melanogaster*. Loss of heterozygosity for wing-hair mutations results predominantly from mitotic crossing over induced in wing anlage cells of larvae, while the reverse mutation of eye-color is due to an intragenic structural change (2.96 kb-DNA excision) in the *white* locus on the X-chromosome. The measurements were performed in a combined mutation assay system so that induced mutant wing-hair clones as well as revertant eye-color clone can be detected simultaneously in the same individual. Larvae were irradiated at the age of 3 days post oviposition with ^{252}Cf neutrons, carbon beam or neon beam. For the neutron irradiation, the RBE values for wing-hair mutations were larger than that for eye-color mutation by about 7 fold. The RBE of carbon ions for producing the wing-hair mutations increased with increase in LET. The estimated RBE values were found to be in the range 2 to 6.5 for the wing-hair. For neon beam irradiation, the RBE values for wing-hair mutations peak near 150 keV/μm and decrease with further increase in LET. On the other hand, the RBE values for the induction of the eye-color mutation are nearly unity in ^{252}Cf neutrons and both ions throughout the LET range irradiated. We discuss the relationships between the initial DNA damage and LET in considering the mechanism of somatic mutation induction.

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INTRODUCTION

There is a need for more information on the biological effects of high-LET radiation, to allow us to predict the risks of radiation exposure in space or during cancer therapy. High-energy heavy ions and neutrons are a component of galactic cosmic rays and constitute a significant part of the biologically effective dose of radiation that astronauts receive during manned space exploration[1–4]. When we consider the genetic effects of high-LET radiations such as cosmic radiations, one of the most important information for us is the relative biological effectiveness (RBE) based on absorbed dose-mutation frequency. Reliable RBE estimates not only give a clear proof of the relative mutational effects of high-LET radiation, but also offer suggestive information for evaluating the genetic hazards of the journey into space. However, the mutagenic effects of these high-LET radiations have not yet been fully studied.

Drosophila melanogaster, called the fruit fly or vinegar fly, is an excellent animal for intensive studies of radiation mutagenesis in reproductive and somatic cells. The RBE of high-LET radiation, especially neutrons, has been extensively investigated in the past using the germ cell mutation system in *Drosophila*[5–9]. These studies have shown that the effectiveness of neutrons to induce recessive lethal mutations is virtually the same for X- or γ -rays, and that neutrons yield high RBE values for chromosomal mutations such as translocations, minute deletions, rearrangements and dominant lethal mutations.

In contrast to germ cell mutations, there are only a few studies on the RBE of somatic mutations in *Drosophila*[10–13]. There are several somatic mutation assay systems in *Drosophila*. These include the wing-spot test[14] and the *white-ivory* (w^i) reversion assay[15].

The mutant wing-spots result primarily from a somatic recombination that involves two recessive genes, *multiple wing hairs* (*mwh*) and *flare hair* (*flr*), which are both located on the third chromosome. In contrast, reversion of the *white-ivory* eye-color mutation results from the excision of a duplicated 2.96 kb-DNA fragment of the *white-ivory* gene on the X chromosome[16]. These two mutation assays seem superior to the conventional recessive lethal mutation test in the germ line for screening for environmental mutagens because of their sensitivity to various mutagens[15,17].

To obtain information on the mutagenic potential of high-LET radiation, we analyzed the production of somatic mutations caused by ^{252}Cf fission neutrons and heavy-particle ions accelerated with a synchrotron in terms of the RBE and LET of radiation sources.

For this purpose, a *Drosophila* strain was constructed that enabled us to detect the two types of mutations simultaneously in the same fly[12,13]. The detection of both wing-hair and eye-color mosaic spot mutations in the same fly allowed us to compare the frequencies of induced mutational events directly, without the ambiguity of separate experiments.

DETECTION OF SOMATIC MUTATIONS AND RADIATION EXPOSURE IN *DROSOPHILA*

Drosophila is a holometabolous insect, meaning that it has larval and adult stages separated by a distinct pupal stage during which metamorphosis occurs. The embryo forms both the larva and islands of cells called imaginal discs, from which the adult skeletal structures form during metamorphosis. These imaginal discs do not start to differentiate until the end of larval development, and only undergo somatic cell division until then. Distinct areas of the adult structure (e.g. wing or eye) are expected to be clones of the progeny cells derived from one single imaginal disc cell. This expectation can be tested experimentally if it is possible to mark genetically individual clones during development in a stable way. When a larva is irradiated with any type of radiation inducing a genetical event in an imaginal disc cell, which is expressed in the adult structure, the phenotypic change based on genetic markers is identified as a marked clone. We applied these two mutation detection systems: the wing-hair mosaic spot assay and the eye-color mosaic spot assay.

Wing-hair mosaic spot assay

In *Drosophila* adult wing, flies homozygous for the recessive marker *mwh* produce 3 or more hairs on each wing epidermal cell, instead of a single smooth hair on each normal cell. The recessive marker *flr* is lethal in the homozygous state. However, when homozygous cells are produced somatically in heterozygous flies, they are viable as long as the clone size is small and produce a flare-shaped hair on the surface of each mutant cell. The loci *mwh* and *flr* are located on the left arm of the third chromosome at map positions 0.3 and 38.8, respectively[18]. Somatic pairing of homologous chromosomes takes place during mitosis. Therefore, it is thus easy to mark clones in heterozygous flies by induced mitotic recombination[14]. When the somatic recombination happen in any position between the centromere and *mwh* locus, the homozygous cell for marker allele is produced. The somatic crossing over between centromere and *flr* locus produce *flr* homozygous and *mwh* homozygous cells through a cell division. Two type of clones are adjoining each other. On the other hand, when the crossing over is between *mwh* and *flr* locus, there appears the *mwh* clone alone.

Eye-color mosaic spot assay

The reversion of the *white-ivory* eye-color mutation is caused by the excision of a duplicated 2.96 kb-DNA fragment of the *white-ivory* gene[16]. As the result of reversion in any germ cell, offspring with this mutation have wild-type colored eyes. When a 2.96 kb-DNA excision occurs in the *white* locus of an imaginal disc cell of a larva with a *white-ivory* allele, a red mosaic spot appears in the compound eye of the adult. A strain with the genotype $Dp[1:1:1:1]w^{i}$ (hereafter as $(w^{I})_{4}$) for the eye-color reversion assay was used throughout this series of experiments. This strain possesses 4 copies of the X-linked eye-color mutation *white-ivory* in a

tandem quadruplication, and shows an increased response to mutagens compared to strain with a single copy of w^{i}[15].

Combining the wing-hair and eye-color assay systems

As described in the previous sections, the eye-color and wing-hair mutation detection assay systems are located on the X and third chromosome, respectively. We installed both systems in one strain of flies. The combined system permitted the detection of both types of somatic mutation in the same fly[12,13]. This method eliminates possible experimental bias in comparing RBE values for different mutations, which may arise from problems of dosimetry or uncontrollable physical conditions.

Radiation exposure and detection of mutations

Females with $(w^{i})_4$ on the X-chromosome and *mwh* on the third chromosome were mated with males with the *flr* allele on the third chromosome, and allowed to egg-lay. Three days later, the larvae were collected from the culture bottles for irradiation. For the details of the high-LET radiation and those standard radiation exposure refer to Yoshikawa et al[12,13]. Carbon ions of LET 13 to 95 keV/μm and neon ion of LET 55 to190 keV/μm were used. Immediately after exposure, all the larvae were kept at 25°C until the adults emerged seven days later. Adult flies typically emerge about 10 days after egg-laying.

The emerged flies were sorted by eye and body color phenotype, and then the males, in which both the $(w^{i})_4$ and *mwh*/*flr* mutation assays are possible, were collected. They were scored for red spot in *white-ivory* eyes and wing-hair mosaic spots, both *mwh* single and *mwh-flr* twin.

RBE-LET RELATIONSHIPS FOR SOMATIC MUTATION INDUCTION

Mutation induction and RBE for ^{252}Cf neutrons compared to ^{137}Cs γ -ray

Somatic reversions of the *white-ivory* gene were detected as mosaic spots composed of wild-type red-colored eye facets in flies exposed to either ^{252}Cf radiation or ^{137}Cs γ-rays at the larval stage. The data presented in the left panel in Fig. 1 for $(w^{i})_4$ males indicate that the mutagenic efficiency of ^{252}Cf radiation was almost equal to that of ^{137}Cs γ-rays for inducing eye-color mosaic spots[12].

The two types of wing-hair mosaic spots are identified as *mwh* single spot and *mwh-flr* twin spots. Moreover, the *mwh* single spots were classified as either large *mwh* spots composed of 3 or more mutant cells or small spots of one or two cells, as recommended by Graf et al[14]. Generally, the large *mwh* spots and *mwh-flr* twin spots originate primarily from somatic crossing over[11,12]. The right panel in Fig. 1 shows the frequency of somatic crossing over, plotted as a function of dose for both types of radiation. ^{252}Cf radiation appeared to be about 6 times more effective than ^{137}Cs γ-rays for inducing wing mosaic spots resulting from crossing over events.

^{252}Cf radiation consists of mixed neutron and γ-ray radiation; 67% of the total tissue dose is

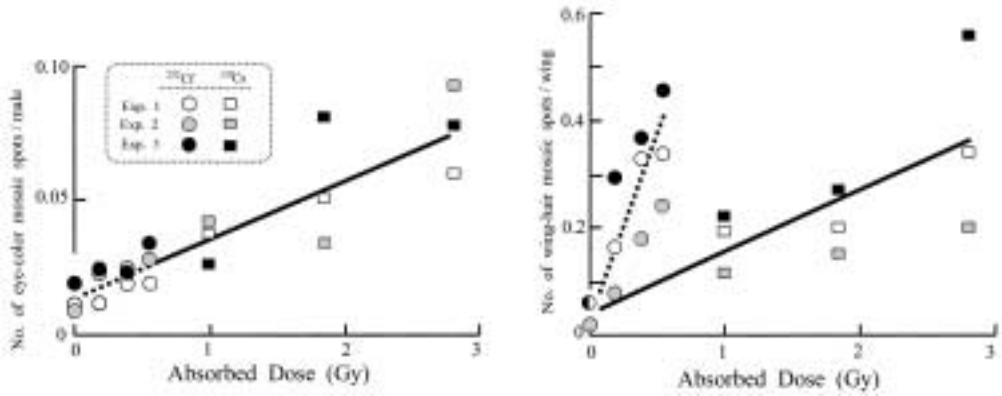

Fig. 1. Induction of eye-color and wing-hair mosaic spots by mixed neutron/γ -radiation of ^{252}Cf or ^{137}Cs γ -rays. The RBE of ^{252}Cf neutrons is 8.5 for wing-hair mosaic spots and 1.2 for eye-color reverse mutations, respectively.

neutrons and the remaining 33% γ-rays[19]. Based on the observed rates of mutation induction, the estimated RBE of ^{252}Cf neutrons was 8.5 for the induction of wing spots and it was 1.2 for the induction of eye spots. Large RBE values with high LET radiation have also been reported for the induction of wing-hair mutations by fission neutrons[10,11].

RBE-LET relationship for two types of somatic mutation induced by carbon and neon ions

The previous experiment revealed distinctly different RBE values for ^{252}Cf neutrons inducing mitotic crossing over and w^i reverse mutations. ^{252}Cf neutrons have higher LET value (average 40 keV/μm) than that of ^{137}Cs γ-rays. This suggests that mitotic crossing over depends on LET, but eye-color reverse mutation does not.

To obtain information on the mutagenic potential of high-energy particles, we analyzed the production of somatic mutations resulting from carbon and neon ion irradiation in terms of the RBE and LET of the particles.

Fruit fly larvae containing both mutation assay systems were exposed to 290 MeV/u carbon ions and 400 MeV/u neon ions generated by the HIMAC (Heavy Ion Medical Accelerator in Chiba) synchrotoron at the National Institute of Radiobiological Sciences, Japan. The experimental procedure was the same as that used in the neutron experiments. Figure 2 shows the relationship between RBE and LET for the induction of wing-hair and eye-color mosaic spot mutations caused by carbon and neon ion irradiation.

It is clear that the carbon beam irradiation with the highest LET ($\cong$95 keV/μm, RBE $\cong$ 6.5) was much more effective than those with lower LET values ($\cong$13 keV/μm, RBE $\cong$ 1.5 and $\cong$ 60 keV/μm, RBE $\cong$ 3.3). In neon beam irradiation, the RBE values for wing-hair mutations peak near 150 keV/μm and decrease with further increase in LET. The RBE for inducing wing-hair mosaic spots shows a strong LET dependence.

In contrast to the case with wing-hair, the induction rates of eye-color mutation by both ion beams are not significantly different from those of X-rays. These data show that high-LET

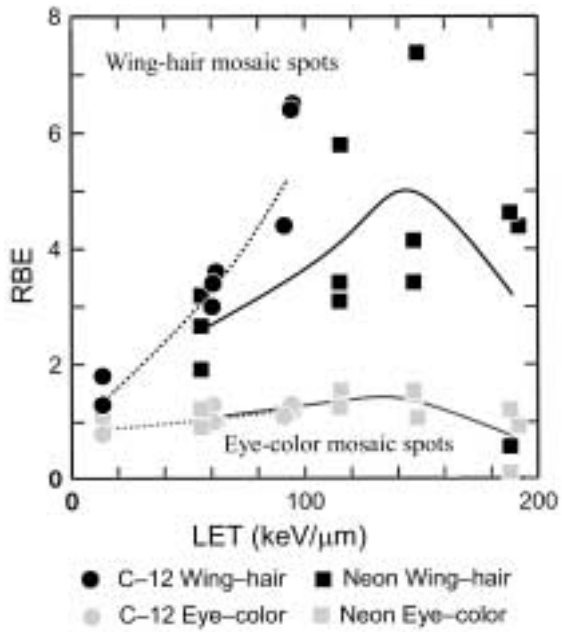

Fig. 2. RBE-LET relationships for somatic crossing over and reversion induction in *Drosophila* irradiated with carbon [13] and neon ions.

radiations are not more effective than X-rays in inducing the eye-color mosaic spot mutations.

LET DEPENDENCE OF MUTATION CROSS SECTION FOR THE TWO TYPES OF SOMATIC MUTATION

The cross section for a given biological event for a given charged particle is the product of the geometrical target cross section and the probability that the event occurs when a particle passes through the target. Therefore, the cross section expresses effect per particle. Figure 3 shows the relationship between the cross section and LET for induction of the wing-hair and the eye-color mutations. For calculation of the cross section, cell number of wing and compound eye imaginal discs when the larvae are irradiated is required. Evaluation of the cell number is executed under the following assumptions.

1. Imaginal disc cell number is proportional to the mutation frequency per absorbed dose by X-rays.

2. Imaginal disc cell number on average follows the following expressions[20].

Wing: $N = 2^{\frac{t}{T_W}}$

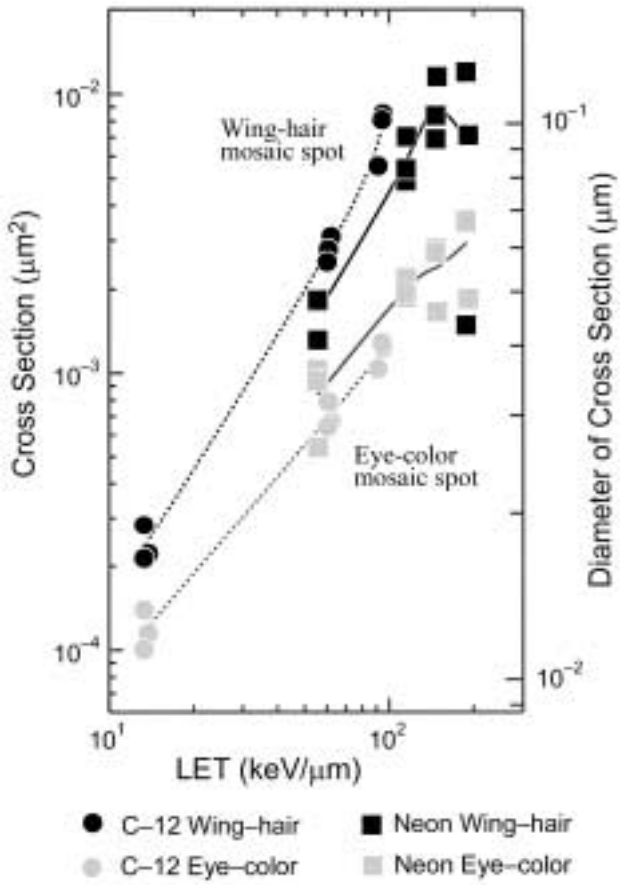

Fig. 3. LET dependence of cross section for mutational events in wing-hair and eye-color. Right scale indicates sphere diameter which has the same cross section.

Eye: $N = 2 \times 2^{\frac{t-16\mathrm{h}}{T_E}}$

Where N is imaginal disc cell number, t is age of the larva, T_w is doubling time of wing imaginal disc cells (8.01 h), and T_E is doubling time of eye imaginal disc cells (7.67 h).

The cross section for the wing-hair increases with LET about proportional to the square of LET and reaches a plateau or drop in the LET region above 100 keV/μm. Fluctuation of the data is large in high LET region, and it is not clear whether the cross section reaches a plateau or drop. If the cross section reaches a plateau, it suggests that the probability that the event occurs when a particle passes through the target reaches unity, and the cross section is almost the same as the geometrical target cross section. If the cross section drops, it suggests that the cell which is passed through by a charged particle is selectively killed and the apparent mutation frequency decreases[21].

The cross section for eye-color is about proportional to the LET. It is paraell to the fact that the RBE value is constant.

The maximum cross sections for wing-hair and eye-color are both about the area of a diameter 0.1 μm sphere. It means that the geometrical size of the target is larger than 0.1 μm. This is very greatly larger than the diameter of the DNA double helix (2 nm), and a little larger than the diameter of chromatin fibre ($\approx$ 25 nm).

DNA DAMAGE AND MUTATIONS CAUSED BY HIGH-LET RADIATION

In these experimental series, we obtained relationships between RBE and LET for the induction of two different somatic mutations by ^{252}Cf neutrons (average LET = 40 keV/μm) , carbon ion beams (applied LET values $\cong$ 13 to 95 keV/μm) and neon ion beams (applied LET values $\cong$ 55 to 190 keV/μm). One mutation involves a mitotic crossing over, detected as wing-hair mosaic spots on the wings, and the second is an intragenic mutation, detected as eye-color mosaic spots on the compound eyes. The assay system permitted the comparison of the two types of somatic mutation without experimental bias.

It is generally accepted that in the trans-heterozygous cell marker system used, all *mwh-flr* twin spots are the descendants of cells that underwent homologous recombination proximal to the *flr* locus. The *mwh* large single spots result primarily from recombination between the *mwh* and *flr* loci or from small terminal deletions covering the *mwh* locus[11,12]. Using a mitotic recombination suppressor system, Schweizer [22] suggested that homologous recombination is the main mechanism for the loss of heterozygosity at the *mwh* locus.

The *white-ivory* eye-color mutation is caused by tandem duplication of a 2.96 kb-DNA sequence in the white locus. Reversion to the wild type is associated with excision of one copy[15,16].

We discuss the possible mechanism(s) by which intracellular DNA lesions may initiate the two types of mutations. The linear dose response seen in these studies for mutations produced by any type of radiation indicates the absence of an intertrack effect. The most commonly observed types of damage to the DNA in a cell following irradiation are single and double strand breaks, base damage and DNA-protein cross-links, resulting from the stochastic nature of radiation interactions. Base damage and single strand breaks are readily repaired within a cell. On the other hand, double strand DNA breaks have been implicated as the initial molecular damage leading to specific genetic changes (chromosome aberration, mutation, instability)[23–26]. The experimental evidence shows that the yield of single and double strand breaks is nearly the same for X-rays and γ -rays in mammalian cells, and is similar to that induced by high LET radiation including neutrons[27–32]. Nikjoo et al[32,33] classified DNA damage resulting from low and high LET radiation according to the complexity and clustering of the damage. It has been suggested that the more complex types of clustered damage are less repairable and hence more likely to produce detectable biological lesions[33,34].

The RBE-LET relationship observed for wing-hair mosaic spot induction suggests that the

DNA damage induced by high-LET radiation is qualitatively different from that induced by low-LET radiation[12,34]. This suggests that the amount of more complex types of DNA damage, such as non-rejoinable or clustered double strand breaks, may increase with the LET value of ions, at least up to 95 keV/μm. Therefore, it is possible that the large *mwh* single and *mwh-flr* twin spots result from more complex types of double stranded DNA breaks[30,33,35].

The origin of the eye-color reversion mutation is less clear. The *white-ivory* eye-color mutation reverts to wild type with the excision of a duplicated fragment in both germ and somatic cells[15,16]. The mechanism responsible for this excision is not well known, but evidence suggests that intra-chromosomal recombination is the main mechanism for w^i reversion[15,36]. However, alternatives such as mispairing of the duplicated sequence at the replication fork[36], gene conversion or unequal crossing over[15,37] cannot be ruled out, as all these processes may lead to loss of the duplicated fragment.

Previously, we speculated that single strand breaks might produce the eye-color mosaic spots because of the small RBE value of ^{252}Cf neutrons[12]. In support of this hypothesis, the antitumor drug 5-fluorouracil and its derivatives, which produce double strand breaks but no single strand breaks[38], did not induce eye-color reversions, although these drugs produced high levels of wing-hair mutations[39]. Kozubek et al[40] found no LET dependence for the induction of forward mutations of *lacI-* in *Escherichia coli* Y_{mel} and $his^- \rightarrow his^+$ reversion in *Salmonella typhimurium* TA102. Experiments with the bacteriophage T4 *amber* mutant using alpha particles do not show LET dependence for the reversion to the wild type[41]. These authors also suggested that a simple form of molecular damage, such as a single strand break or base modification, might be responsible for the reversion to the wild type. On the other hand, mitotic recombination processes must also be considered as a mechanism for the reversion. To elucidate the mechanism(s) for inducing these mutations it is necessary to characterize the molecular nature of the damage further both experimentally and theoretically.

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