

Somatic Mutation in Larvae of the Silkworm, *Bombyx mori*, Induced by Heavy Ion Irradiation to Diapause Eggs

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In order to investigate whether eggs of the black-striped strain (P^S) of the silkworm, *Bombyx mori*, represent an appropriate model for estimating the biological effect of cosmic radiation, radiosensitivity of the eggs against X-rays and heavy ion particles was examined as ground-based experiments. The exposure of diapause eggs to X-rays or heavy ion particles resulted in somatic mutations appearing as a white spot on the black integument during larval stage. Irradiation of non-diapause eggs with X-rays demonstrated a significant difference in frequency of the mutation between fractionated and single administration doses, but no difference was observed in diapause eggs. Incidence of the mutation as induced by carbon ion beams for 15-day old eggs was higher for eggs that had been kept at 15°C than those kept at 25°C. Neon beam irradiation of diapause eggs displayed dose- and linear energy transfer (LET)-dependent effects, causing a maximal rate of the mutation at 150 keV/ μm . These results confirm that *B. mori* eggs represent valid models for estimating the biological effects of cosmic radiation.

INTRODUCTION

As to acute and late biological effects of long-term space flight, and developing biodosimetry for the assessment of health risks associated with space radiation exposure¹⁾ is critical to safe human occupation of the International Space Sta-

tion (ISS). Responses of organisms to the space environment have been analyzed by loading various organisms onto space shuttles. Such experiments have examined the synergistic effects of cosmic radiation and microgravity on larvae of *Drosophila melanogaster*²⁾, embryos of *Carasius morosus*³⁾ and embryos of the silkworm, *Bombyx mori*⁴⁾.

In 1997, the authors loaded *B. mori* eggs onto the US space shuttle/Atlantis (STS-84) to examine the effects of cosmic radiation on embryonic and post-embryonic development. This experiment demonstrated abnormal embryonic reversal in addition to abnormal fusion between the 4th and 5th segments⁴⁾. Silkworm eggs were used as biological models for the following reasons: 1) differentiation, diapause, metamorphosis, and genetic characteristics are well understood for *B. mori*; 2) embryos can be maintained in diapause state for at least 2 years by controlling storage temperature of the eggs⁵⁾ and 3) large quantities of silkworm eggs can be loaded onto the ISS to obtain meaningful and statistically significant data regarding survival ratios, mutations, abnormal morphogenesis, and specific gene mutations.

Since the previous space flight experiment⁴⁾, simple and convenient methods of assessing the biological effects of

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cosmic radiation have been requested. Regarding the effect of irradiation on morphological expression of the silkworm, irradiation of black-striped (P^S) larvae with γ -rays⁶⁾ or neutrons⁷⁾ induces a white spot on the black integument due to a mutation in the dominant P^S gene that prevents synthesis of melanin⁸⁾ in epidermal cells.

The present study examined whether irradiation of silkworm eggs with X-rays or heavy ion particles would induce white spots on the integument of larvae, and if the mutation could be used as a biological index for estimating exposure to cosmic irradiation.

MATERIALS AND METHODS

Sample materials

A black-striped strain of *B. mori* with the dominant gene (P^S) was utilized.

Exposure of eggs to X-rays or heavy ion particles

Eggs of P^S/p heterozygotes were exposed to either X-rays (200kVp) at the Radiation Biology Center, Kyoto University, or heavy ions, including carbon-12 (290 MeV/u) or neon-20 ion beams (400 MeV/u) accelerated by the Heavy Ion Medical Accelerator at Chiba (HIMAC) synchrotron at the National Institute of Radiological Sciences, Japan. Detailed methodology and experimental conditions of the heavy ions irradiation and the dosimetry have been described elsewhere^{9,10)}. In brief, the energy fluence of the heavy ion beams was measured by a plastic scintillator, and LET was measured with a proportional counter filled with a tissue equivalent gas. The thickness of the lucite absorber plate (range filter) in front of the egg samples was adjusted to obtain the desired LET values approximately 13, 50, or 80 keV/ μ m for carbon ion, and 30, 70, 140, or 190 keV/ μ m for neon ion. Radiation dose at the sample portion was adjusted by multiplying the fluence by the stopping power in water. Dose to the sample was controlled by a parallel plane ionization chamber. The dose rates of the heavy ions were 0.1 – 1 Gy/min for the entire experiments. All irradiation was performed at room temperature.

Detection of somatic mutations

Diapause eggs irradiated with X-rays or heavy ion particles were kept for 2 or 3 months at 5°C to terminate diapause, and then incubated for 12 days at 25°C to obtain newly hatched larvae. Larvae were reared until the 3rd day of 5th instar. The number of the larvae with a white spot (Figs. 1a and 1b) was then counted. Incidence of the somatic mutation was calculated by dividing the number of larvae

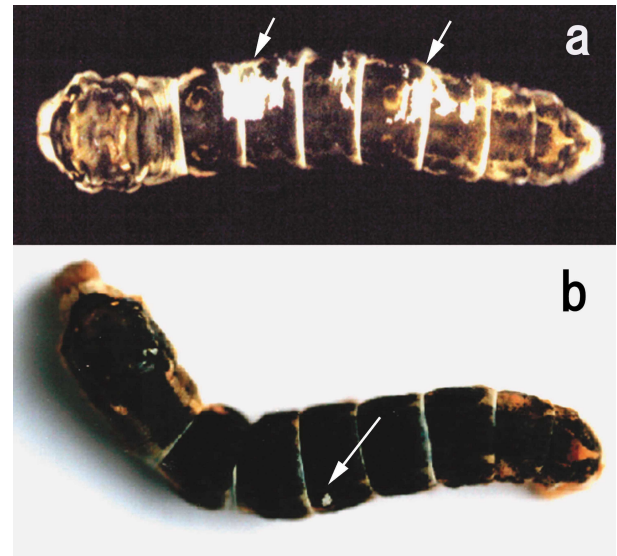


Fig. 1. Somatic mutation appears as a white spot (arrows) on black integument of 5th instar larvae from X-ray or heavy ion beam irradiated eggs of the silkworm, *Bombyx mori*. This white spot is caused by mutation of the P^S gene.

with a white spot by the total number (300) of larvae. Spontaneous mutation was not detected in all experiments as back ground.

RESULTS

Somatic mutation by X-ray irradiation of early developmental eggs

In diapause-destined eggs at 25°C, embryos proceed towards stage 7 (Telson formation with 18 segments) for about 3 days, and then stop development¹²⁾. From oviposition through stage 7, eggs were exposed at each stage to X-rays, and newly hatched larvae were reared to investigate the incidence of somatic mutation on the integument of 5th instar larvae (Fig. 2). Irradiation of eggs with 1.0 Gy of X-rays at 2 or 5 h after oviposition induced somatic mutation in about 5%, including mosaic larvae (Fig. 1a) in addition to black-striped larvae with a white spot (Fig. 1b). With irradiation from 10 h after oviposition, the only mutations observed were larvae with one white spot. Thereafter, incidence of mutation increased with embryonic development during the initiation of diapause, but decreased gradually as diapause state proceeded. Conversely, when some of these eggs were treated with HCl solution to avoid entrance into the diapause state, incidence of somatic mutations increased compared to that in diapausing eggs.

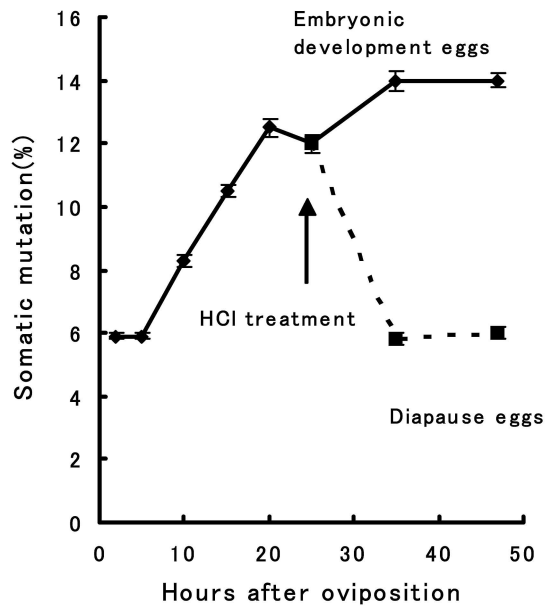


Fig. 2. Changes in the frequency of somatic mutation in 5th instar larvae from X-ray irradiated eggs during early embryogenesis. In diapause-destined eggs (diapause eggs), Eggs gradually stop the embryonic development about 24 h after oviposition, and enter into diapause state. Around this time, eggs are treated to hatch artificially using heated HCl (sp. gr., 1.075, 46°C, 5 min) to continue embryogenesis for about 10 days (embryonic developing eggs). Eggs were irradiated with 1.0 Gy of X-rays at the indicated times.

Effect of single and repeated radiation on incidence of somatic mutation

Diapause-destined eggs were divided into two groups. One group was used to enter diapause state (diapause eggs), and the other was treated with HCl at about 25 h after oviposition to continue embryonic development (non-diapause eggs).

1) Embryonic development egg of non-diapause eggs

After treatment with HCl, non-diapause eggs were kept at 15°C to maintain the developmental stage between stage 6 (formation of ectoderm and mesoderm) and stage 8 (just before diapause I)¹¹⁾. Some of these eggs were exposed once or twice at interval of 1 day to 0.5 Gy of X-ray radiation. The remaining eggs were exposed to a single dose of 1.0 Gy of X-ray radiation. Incidence of somatic mutation in 5th instar larvae was compared between different exposure groups (Fig. 3a), and was significantly lower in eggs exposed to the single dose than in those exposed to the single-administration dose.

2) Diapause eggs

Diapause eggs were initially kept at 25°C for 2 days, and then at 5°C for 70 days. These eggs were exposed to X-rays of either a single dose of 1.0 Gy or repeated doses of 0.33 Gy for one, or two or three times with an interval time of one week each (Fig. 3b). Repeated exposures resulted in an increased incidence of somatic mutation. Incidence of mutation following the repeated exposure (0.33 Gy \times 3) was almost identical to incidence following the single exposure (1 Gy \times 1).

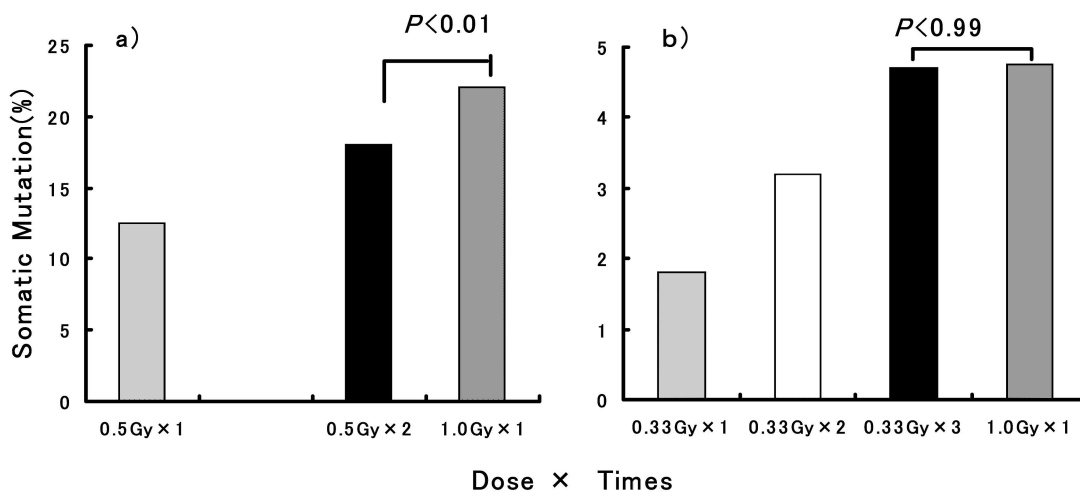


Fig. 3. Effects of single or fractionated dose X-ray irradiation on the incidence of somatic mutation, and additivity of the dose. a) Eggs were kept at 25°C for first 2 days after oviposition, and then at 5°C for 70 days. These eggs were irradiated 1, 2 or 3 times at intervals of one week with 0.33 Gy of X-ray, or with 1.0 Gy of X-rays as a single-administration dose. b) Diapause eggs were treated with 6 N HCl at 46°C for 5 min and then transferred to 15°C. The eggs were irradiated 1 or 2 times at intervals of one day with 0.5 Gy of X-rays, or with 1.0 Gy of X-rays as a single-administration dose.

Effects of storage temperature on incidence of somatic mutation following irradiation with carbon ion beams

Polyols (sorbitol and glycerol) are well known to accumulate spontaneously in diapause eggs of the silkworm⁷⁾, and the concentration of polyols fluctuates with progression of diapause and is affected by temperature. In addition, polyols influence damage to DNA molecules in aqueous solution due to radicals by irradiation¹²⁾. We therefore examined the effect on incidence of the somatic mutation of varying polyol content, by storing eggs under different temperatures. Eggs were kept at 15°C or 25°C for 15 days after oviposition, then irradiated with 0.5 Gy or 2.0 Gy of carbon ion particles with 13 or 80 keV/ μ m, respectively (Fig. 4).

Incidence of somatic mutation was significantly lower in larvae from eggs kept at 25°C than in those from eggs kept at 15°C. Sorbitol content was 5.7 μ mol/100 eggs and 3.2 μ mol/100 eggs in eggs kept at 25°C and 15°C, respectively. This result suggests that differences in polyol content in eggs exert an influence on the incidence of somatic mutation following irradiation.

Relationship between incidence of somatic mutation and dose or LET of heavy ion particles

Exposure of diapausing eggs to carbon ion particles increased incidence of the mutation in a dose- (0.5–2.0 Gy) and LET-dependent (25–80 keV/ μ m) manner. A dose of 2.0

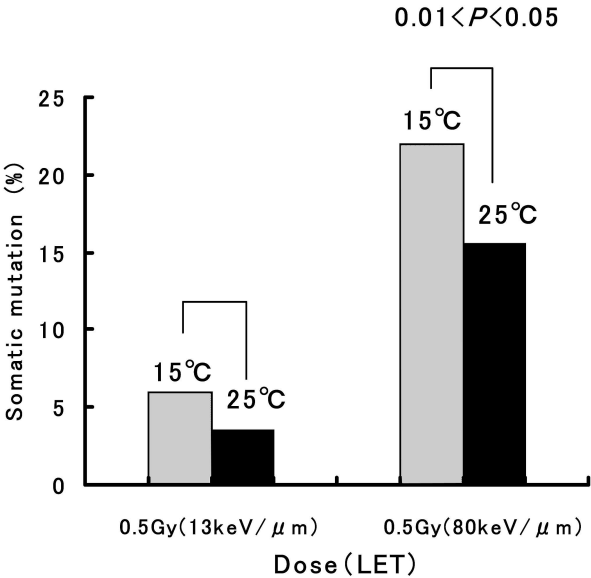


Fig. 4. Effect of different temperatures on the incidence of somatic mutation in larvae hatched from carbon irradiated eggs. Eggs were kept at 25°C or 15°C for 15 days after oviposition, and then irradiated with 0.5 Gy (13 keV) or 2.0 Gy (80 keV) of carbon ion particles. Values were calculated using χ^2 -test.

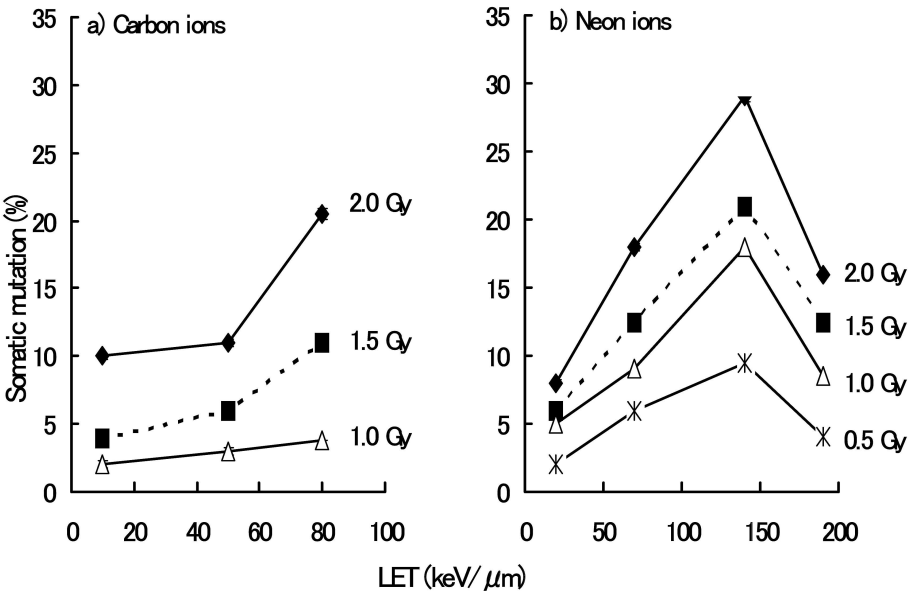


Fig. 5. Dependency of the incidence of somatic mutation on both dose and LET of carbon and neon ion particles. Eggs were kept at 25°C for the first 2.5 days after oviposition, then transferred to 5°C. Two months later, these eggs were irradiated with carbon ion particles (290 MeV/u). b) Eggs were kept at 25°C for the first 15 days after oviposition, then transferred to 0°C. This maintains an almost constant sorbitol content for at least 200 days, meaning that eggs remain in the diapause state⁵⁾. These eggs were irradiated using neon ion particles (400 MeV/u).

Gy of carbon ion particles with an LET of 80 keV/ μ m induced only 20.5% incidence of the mutation (Fig. 5a). In order to examine the dependency of mutation incidence on LET, neon ion particles with more than 80 keV/ μ m were used. Diapausing eggs, which were kept continuously at 0°C, were irradiated using a neon ion beam with dose and LET as shown in Figure 5b. Mutation incidence increased in a dose-dependent manner, and in an LET-dependent manner up to 150keV/ μ m. However, mutation incidence decreased substantially under irradiation at 200 keV/ μ m.

DISCUSSION

Exposure of larvae of the black-striped strain of *B. mori* to γ -rays or neutrons has been shown to induce a white spot on the backs of larvae as a somatic mutation^{6,7}. The present work confirmed that exposure of P^S eggs to X-rays or heavy ion particles such as carbon or neon ions induced the same somatic mutation.

In this system utilizing the P^S gene as an index of radiation exposure, incidence of somatic mutation was clearly dependent on both dose and LET. The P^S gene exists at 0.0 near the telomere of the second chromosome of the silkworm¹³. Even if a reparable cut occurs in this portion, other genes are able to retain functionality. P^S gene is therefore considered the most convenient marker for examining the LET-dependency of complex effects in damage of chromosomal DNA following irradiation with ion particles.

In wing cells and body hair of *D. melanogaster*¹⁴ and embryo-derived hamster cells^{15,16}, the LET inducing the highest incidence of mutation is around 100 keV/ μ m. Mutations for eye color in *D. melanogaster* are not dependent on LET, but mutation in wing cells is¹⁴. In contrast, the LET inducing the highest incidence of somatic mutation in diapause eggs was around 150 keV/ μ m. Diapause eggs of the black-stripe strain therefore represent effective biological models for examining the effects of cosmic radiation with LET higher than 100 keV/ μ m.

The sensitivity of diapause eggs was higher than that of non-diapause eggs (Fig. 2). In addition, sensitivity was lower in eggs kept at 25°C than those kept at 15°C (Fig. 4). Sorbitol content in the former was lower than in the latter. These results could be caused by sorbitol, which accumulated in *B. mori* diapause eggs. Further, sorbitol and glycerol content fluctuates within diapause processes such as initiation, maintenance and termination. Polyols play a role in lowering the sensitivity of eggs to irradiation. Investigation of the sensitivity accompanying these processes is necessary, because diapause eggs will be loaded into the ISS for

at least 6 months, during which time the eggs will pass through all the processes of diapause.

Diapause eggs exposed to X-rays displayed significant differences in mutation prevalence between the repeated (0.33 Gy \times 3 times) and single (1.0 Gy \times 1 time) doses. However, no differences were observed for non-diapause eggs, which underwent embryonic development. Therefore, diapause eggs may not possess the ability to repair DNA strands damaged by irradiation. This conclusion is further supported by the fact that diapause eggs stop cell division of the embryo at G2 phase¹⁷.

According to the basic ground experiments described in the present paper, *B. mori* eggs from the black-striped P^S strain represent one of the most useful tool yet found for examining the biological effects of cosmic radiation using the ISS. In addition, the present study provides a large amount of basic data for radiobiology of *B. mori* diapause eggs.

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