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REVIEW

Why Genetic Effects of Radiation are Observed in Mice but not in Humans

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Genetic effects from radiation have been observed in a number of species to date. However, observations in humans are nearly nonexistent. In this review, possible reasons for the paucity of positive observations in humans are discussed. Briefly, it appears likely that radiation sensitivity for the induction of mutations varies among different genes, and that the specific genes that were used in the past with the specific locus test utilizing millions of mice may have simply been very responsive to radiation. In support of this notion, recent studies targeting the whole genome to detect copy number variations (deletions and duplications) in offspring derived from irradiated spermatogonia indicated that the mutation induction rate per genome is surprisingly lower than what would have been expected from previous results with specific locus tests, even in the mouse. This finding leads us to speculate that the lack of evidence for the induction of germline mutations in humans is not due to any kind of species differences between humans and mice, but rather to the lack of highly responsive genes in humans, which could be used for effective mutation screening purposes. Examples of such responsive genes are the mouse coat color genes, but in human studies many more genes with higher response rates are required because the number of offspring examined and the radiation doses received are smaller than in mouse studies. Unfortunately, such genes have not yet been found in humans. These results suggest that radiation probably induces germline mutations in humans but that the mutation induction rate is likely to be much lower than has been estimated from past specific locus studies in mice. Whole genome sequencing studies will likely shed light on this point in the near future. © 2018 by Radiation Research Society

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INTRODUCTION

Radiation exposures are known to induce various types of DNA damage, which if not properly repaired, may lead to cell death or mutation induction. If the mutations occur in germ cells, they may lead to increased risks of hereditary diseases in the offspring. If they occur in somatic cells, they may lead to an increased risk of cancer. Studies of fruit flies to examine the genetic effects of radiation have a long history. In 1927, Muller found that X-ray radiation could induce mutations in the germ cells of *Drosophila melanogaster* (*1*). Muller won the Nobel Prize in 1946. One important aspect of his accomplishments was that he created a mutant strain that had a large inversion on the X chromosome. In the germ cells of F1 females born to irradiated sires or dams and bearing such an inversion under heterozygous conditions, meiotic recombination leads to dicentric formation and causes lethality in these cells. Therefore, recessive lethal mutations may persist on the irradiated X chromosome. Without this inversion, recessive lethal mutations may either stay on the irradiated X chromosome or move to the nonirradiated X chromosome after a meiotic recombination, which would make it impossible to trace the potentially induced recessive lethal mutations in the F2 generation.

STUDIES WHICH UTILIZED PHENOTYPIC MARKERS

Specific Locus Tests in Mice

In the post-war era after WWII, the world witnessed the start of the Cold War, the threat of nuclear warfare, and the increased use of radiation in the medical and industrial fields. Since relying solely on insect data to assess radiation risks in humans did not appear to be prudent, large-scale mouse genetic studies were initiated in the late 1940s, primarily in the U.S. (at Oak Ridge National Laboratory), and less extensively in the UK (at MRC in Harwell) and in what was then West Germany (at GSF in Neuherberg). In those days, cancer risks had not yet been well recognized as the most important hazard resulting from radiation exposures. The basic method that the specific locus test (SLT)

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uses is shown in Fig. 1A. In the U.S. system, seven loci that were related to coat color or ear shape were selected (*2*), while in UK, six loci were selected (*3*), one of which was common to both systems [see refs. (*4, 5*) for reviews]. Homozygous mice bearing the six or seven recessive marker genes were used as a tester strain (white mice). The tester mice were mated with irradiated wild-type mice to obtain offspring (the F1 generation). All the offspring are principally heterozygous for the selected specific loci; i.e., they inherited one mutant allele from the tester strain (*a*) and one wild-type allele from the irradiated parents (+), and are thus phenotypically wild type. However, on rare occasions where the irradiated wild-type alleles were mutated ($+^{mut}$), offspring with mutated coat colors were detected. Note that the mouse genetic studies primarily focused on germline stem cells in males (spermatogonia cells). In females, mature and maturing oocytes were the main target cells studied because, although resting oocytes are more relevant to human exposure conditions, mouse immature oocytes are highly vulnerable to radiation and die, while this is not the case in humans. A large fraction of the studies to be described below are therefore related to these germ cells.

It took more than 10 years to obtain results after testing more than several million mice (Fig. 1B). Here we see that the mean mutation induction rate per locus increased with an increase in the radiation dose, and protracted exposures gave rise to lower induction rates compared to results from acute exposures, if the total doses were the same. Although not shown in Fig. 1, it is also recognized that resting oocytes, although highly sensitive to killing from exposure, are highly resistant to mutation induction (*6*), and male post-meiotic cells are more mutable than pre-meiotic cells (*5*), as previously shown in *Drosophila* mutagenesis studies. Furthermore, it is clear that the dose range used was nearly 1/10 of that used for *Drosophila* mutagenesis studies, which underscores the importance of not relying on insect data for radiation protection purposes in humans. All this information was obtained in the 1960s and 1970s.

Other Genetic Effects of Radiation in Mice

Genetic effects from radiation, other than mutation induction at specific loci, are represented by tumor frequencies and life shortening in the offspring. Two studies on lifespans have been reported, however, in neither one was this effect found (*7, 8*). On the other hand, at least five studies on tumor or cancer frequencies in offspring have been reported. In four of the five studies, no significant effects of parental exposure to radiation (*7–10*) were found, while in one study, it was reported that the tumor frequency in the offspring increased with an increase in the radiation dose delivered to the parents (*11*). The latter study was reported in 1982, however, progress on the mechanisms involved in these observations has not materialized (no molecular changes related to the effect of radiation have been reported). Since the onset age of tumors is not

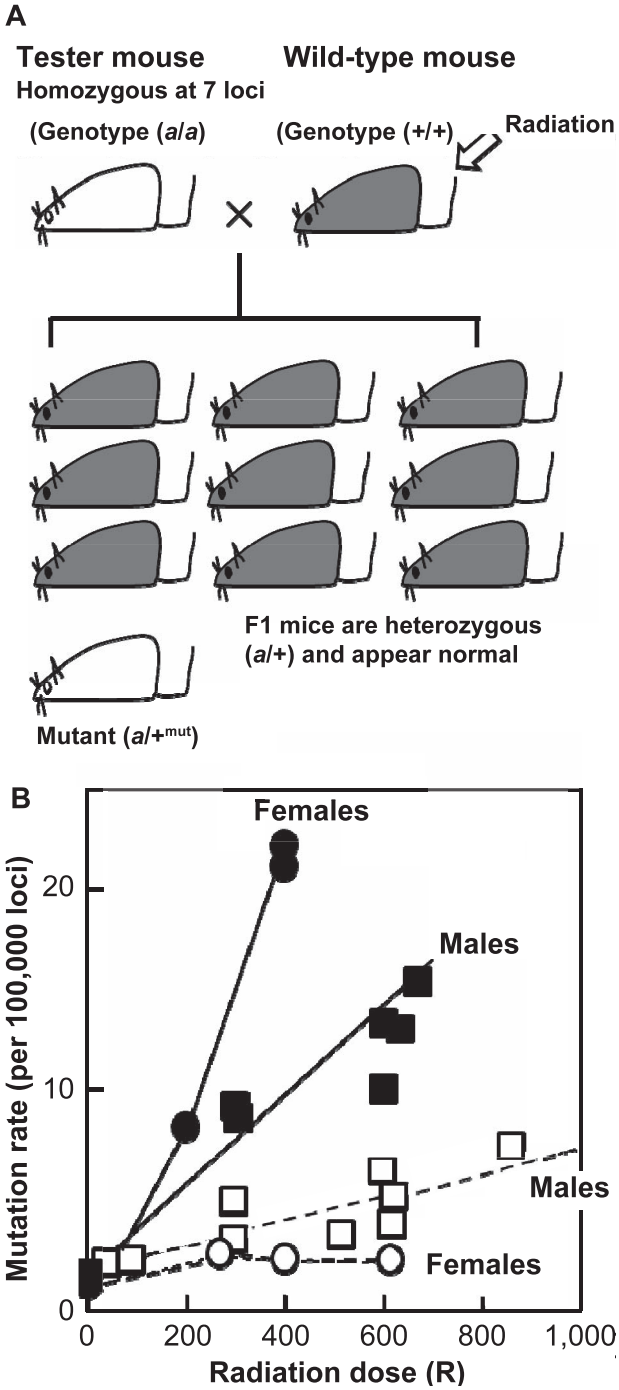

FIG. 1. Panel A. Methods used in the mouse-specific locus test. Preconception radiation exposure to the wild-type mouse could cause a germline mutation in target loci, as shown by "$+^{mut}$", which may result in a phenotypic change in the offspring (white mouse). Panel B: The results. Filled and open symbols indicate results from acute and protracted exposures, respectively. Males represent the results in F1 derived from irradiated spermatogonia, and females represent those derived from irradiated mature or maturing oocytes (*5*). 100 R = ~1 Gy radiation.

accelerated in offspring born to irradiated parents when compared to that of the control animals, the results do not support the hypothesis that irradiation of male or female germ cells induced mutations in one of the tumor suppressor

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TABLE 1
Population Size of Study Subject Groups in the ABCC-RERF Genetic Studies (*13*)

End point	No. of subjects	Study period
Birth defects	77,000	1948–1954
Sex ratio	140,000	1948–1966
Chromosome aberrations	16,000	1967–1985
Electrophoretic variants of blood proteins	23,000	1975–1984
Mortality	75,000	1946–ongoing
Cancer incidence	75,000	1958–ongoing
DNA studies	1,000 families	1995–ongoing

TABLE 2
Genetic Effects of Radiation on the Offspring of Childhood Cancer Survivors (*23*)

Genetic disease	Survivor offspring (n = 6,129)	Sibling offspring (n = 3,101)
Cytogenetic abnormality	7 (0.1%)	6 (0.2%)
Single-gene (Mendelian) disorder	14 (0.2%)	8 (0.3%)
Simple malformation	136 (2.2%)	97 (3.1%)
Total	157 (2.6%)	111 (3.6%)

Note. The mean gonadal doses were 0.46 Gy for male patients and 1.26 Gy for female patients.

genes. Because the strains of mice used by Nomura (*11*) are different from those used in other studies, ''the results may be a specific property of the mouse strains used'' (*11*). If this were the case, then the results cannot be extended to humans because each inbred strain of mice corresponds to a single individual human (humans are clearly outbred creatures or genetically heterogeneous).

Studies in Humans

Genetic studies on the offspring of atomic bomb survivors were initiated soon after the end of WWII in the 1940s. The first study was concerned with birth defects (malformation, stillbirth and perinatal death). Subsequent studies were related to sex ratios, chromosome abnormalities, rare electrophoretic variants of blood proteins, cancer mortality and cancer incidence, etc. In none of these studies was there an observed effect of parental exposure to radiation [see refs. (*12, 13*)]. It should be mentioned here that studies on the sex ratios and electrophoretic variants of blood proteins were not appropriate for detecting the mutagenic effects of radiation based on current scientific knowledge. Specifically, regarding the sex ratio study, maternal exposure to radiation was known to decrease the male-to-female ratio in the offspring of *Drosophila*, since the irradiated X chromosome is inherited by both male and female offspring, while males would die if they carry an X chromosome containing a recessive lethal mutation and females would not die, given the presence of another nonirradiated X chromosome. The same effect was expected to occur in humans. However, although the sex-chromosome composition in females is the same XX in both *Drosophila* and mammals, the two X chromosomes are active throughout the life in *Drosophila*, which was later found not to be the case in mammals; specifically, one of the two X chromosomes is inactivated in an early stage of development (lyonization). This means that an X-chromosomal recessive lethal mutation may be expressed in approximately 50% of the embryonic cells of a female zygote, which is likely to be detrimental to the embryos. Therefore, it is understandable that no firm evidence was obtained for the induction of X-linked recessive lethal mutations in mice (*14, 15*). Another source of the possible species differences is that the proportion of the X chromosome to the whole genome is much larger in flies (approximately 20% in males), compared to only 5% in humans. As for the second issue, although the method itself was appropriate in those days, ionizing radiation is not effective in inducing base-change mutations that will give rise to electrophoretic variants. The results of birth defects are presently under reevaluation. Studies on mortality and cancer incidence as well as genomic alterations are still ongoing. Table 1 summarizes the size of the study populations for each project. The estimated mean dose to the gonads of epidemiologic study subjects is 264 mGy (*16*).

Other study populations in addition to the offspring of A-bomb survivors are the offspring of childhood cancer survivors. Radiotherapy treatment for cancer often uses exposures of 2 Gy, which are repeatedly (25–30 times) delivered to the tumors. Because radiation scatters when it hits any material, exposure of the gonads to substantial amounts of scattered radiation is often unavoidable. For instance, ovaries or testes may receive over 20 Gy when Wilms tumors are treated, given that the site of this cancer of the kidneys is closer to the ovary or testes than organs that are involved in other childhood cancers, such as brain tumors or retinoblastoma. Studies on the offspring of such cancer patients have been reported (*17–24*), and thus far any genetic effects of parental exposures to radiation remain undetected. Table 2 represents a summary of one report.

Mutations at Repeat Sequences in the Genome

The only studies that showed a possible genetic effect of radiation in humans are mutation analyses at minisatellite loci [e.g., (*25*)], which are known as DNA fingerprints because the number of repeat sequence blocks is highly variable among individuals. However, these results are not widely accepted because similar studies failed to reproduce these results (*26–28*). While similar repeat sequences are present in the mouse genome, they are termed expanded simple tandem repeats (ESTR) and are considered to be different entities from human minisatellites. Radiation exposures seem to increase the mutation frequency at the ESTR loci, but not all of the results are concordant (*29*). In addition, microsatellites, which consist of shorter units of repeat sequences, are also studied in both mice and humans,

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TABLE 3
Brief Comparisons of Radiation Effects on Heredity between Mice And Humans

End point	Mouse	Human
Specific locus mutation	Yes (*2–5*)	No study
Life shortening	No (*7–8*)	No (*16*: ongoing)
Tumor frequency	No (*7–10*); yes (*11*)	No (*16*: ongoing)
Malformation	Yes and No[a] (*11, 32*)	No (*12, 13*)
ESTR/minisatellites	Yes but controversial[b] (*29*)	Yes (e.g., *25*); no (*26–28*)
Microsatellites	No (*31*)	No (*30*)
Genomic CNV[c]	Yes but weak (*33, 34*)	No study

Notes. ''Yes'' indicates positive and ''No'' nonsignificant results. Dominant lethal mutation is not included here because it is only detectable in mice and not in humans (objective collection of human abortion data was difficult).

[a] The results varied not only between irradiations of female vs. male germ cells, but also between irradiations of pre- vs. post-meiotic cells in males, as well as pre- vs. postnatal observations.

[b] While one group showed the premeiotic stage was the sensitive stage, the other group showed the postmeiotic stage was the sensitive stage.

[c] CNV = copy number variation.

but the mutation frequency does not appear to increase after radiation exposures (*30, 31*).

The above-mentioned reports provide the basis underlying the statement that the ''genetic effects of radiation are observed in mice but not in humans''. A brief summary is given in Table 3.

STUDIES ON DNA

A Path Toward Testing Many Genes Simultaneously

From the mouse SLT study, it was learned that the mutation induction rate per locus is on the order of 10^{-5} per Gy. Detecting such a low frequency with genetic studies in animals requires considerable resources, restricting the use of this approach. These conditions were considered to be surmountable if a large number of genes could be tested in each animal, and it would then become possible to conduct genetic studies in modestly equipped laboratories. In a step toward achieving this goal, the two-dimensional gel electrophoretic (2DE) method using ^{32}P-labeled DNA was chosen at the Radiation Effects Research Foundation (*35, 36*). With this technique, ^{32}P-labeled DNA fragments are visualized as dark spots on autoradiograms. The intensity of each spot was determined by a computer-assisted scanner, and this value was used to find spots with half the density of controls, and these spots were deletion candidates. Subsequently, the isolated DNA from the normal spot could then be used as a probe to analyze structural changes associated with the deletion. The 2DE method could detect nearly 1,000 DNA fragments from each animal. Therefore, examining 100 mice would be equivalent to testing 100,000 genes (or 200,000 genes if we consider that each spot is composed of both maternal and paternal alleles).

Using the 2DE method, 500 mouse offspring derived from 4 Gy irradiated spermatogonia cells and 500 offspring of the control mice were screened. Because 500,000 ^{32}P-labeled DNA fragments were screened for each group, it was expected that approximately 50 mutations would be induced if a 4 Gy exposure increased the mutation frequency to approximately 10×10^{-5} per locus, as would be expected from the mean mutation induction rate from the mouse SLT studies (Fig. 1). Contrary to expectations, only 5 mutations were detected in the irradiated group and one in the control group. Because the 2DE method was able to detect a deletion as small as 6 bp (CTCTCT) at a microsatellite locus (*37*), it is difficult to imagine that the study overlooked as many as 45 deletions (the difference between the expected and observed) or 90% of the small deletions. The results of this study suggested that radiation responses for the induction of mutations are probably highly variable among genes and that the mean induction rate is low. In other words, testing, e.g., 10 genes from 100,000 animals could provide different results from testing 1,000 genes for 1,000 mice even though the total number of genes tested are the same.

A New Approach Utilizing Genome Information

A recent major step in biology is whole genome sequencing. With improvements in the methodology, it became reasonable to propose a study that could compare the genomes of both parents and their offspring. Such a study would provide a decisive explanation about the genetic effects of radiation in humans. However, currently, whole genome sequencing techniques are reasonably effective at detecting base-change mutations, but are not yet good enough to detect copy number changes (insertions and deletions) (*38*). Currently, one of the best methods to detect deletion mutations would be to use the array-based comparative genomic hybridization (CGH) method (see Fig. 2 for the outlines). One problem associated with the array-based approach is that the hybridization process may vary among various probes, and thus an altered green/red ratio at a single isolated probe could be caused mainly by noise. To avoid this problem, probes are lined up to conform with their locations on the chromosomes, and genomic regions are sought, wherein probes show altered green/red ratios at two or more adjacent locations as a cluster. Finally, each candidate for copy number variation (CNV) must be tested by sequencing for confirmation.

Results of Array-CGH Studies

Two reported studies have utilized the array-CGH technique to screen deletions at more than one-million sites in the genomes of offspring born to male mice irradiated at the spermatogonia stage (*33, 34*). In both studies, it was

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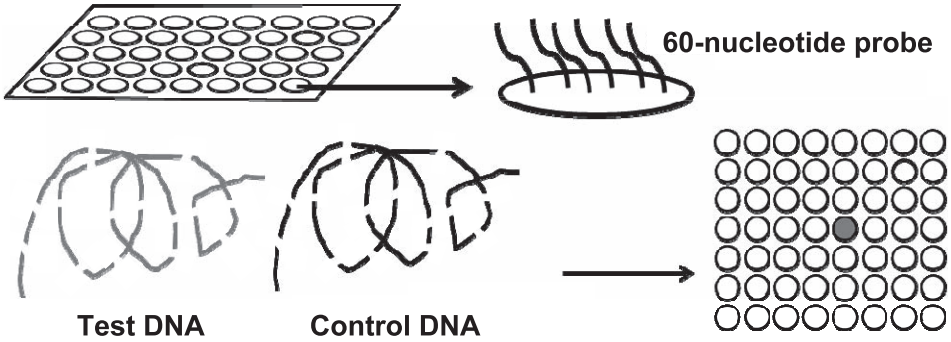

FIG. 2. Detection of deletions and duplications using microarrays. Red and green DNA are indicated in gray and black here, respectively. The two DNA samples were mixed and hybridized to the microarray. Each circle (lower right side) represents probes, which consist of synthesized single-strand DNAs (~60 mers), each of which corresponds to a different part of the genome. If the copy number of red and green DNAs were the same, each probe would show the same yellow color (open circle), whereas if a deletion were present in the red or green DNA, the corresponding probe(s) or spots would exhibit a greenish or reddish yellow color, respectively (filled circle).

found that only a small number of deletions could be detected. Below is a description of a study from our laboratory (*34*).

If it is assumed that any part of the genome can respond to radiation as did the mean of seven loci in the SLT studies, exposure to 1 Gy is expected to give rise to nearly one deletion per F1 genome. Namely, as the mean mutation induction rate was $1–2 \times 10^{-5}$/locus per Gy, and the genome contains approximately 25,000 protein coding genes, the expected number of deletion mutations in any of these genes would be $(1–2 \times 10^{-5}) \times 25{,}000 = 0.25–0.50$ per Gy. Furthermore, since small-size deletions may occur in noncoding regions (e.g., intervening sequences), which comprise approximately 75% of the genome, the expected number of deletions per genome might be doubled, or 0.5–1 deletions/genome per Gy.

Again, quite contrary to expectations, only five deletions were found among 100 offspring born to 4 Gy irradiated sires and one deletion among 100 control mice. Figure 3 shows two examples of the deletions; one was the smallest (1.7 kb) and the other was the largest (4,664 kb) (the scales of the x-axes are not the same). If the frequency observed in the control group was subtracted, the mutation induction rate is approximately 1 deletion/100 genomes (or 0.01 deletions/genome) per Gy. Compared to the expected numbers of 0.5–1 deletion/genome per Gy, the difference between the expected and observed numbers is 50–100-fold. This difference may be reduced to one-half if one assumes that nearly one-half of the induced deletions were too small to be detectable with the array-CGH method. However, the difference cannot be further reduced by assuming that small mutations were the predominant-type of deletions, because many mutagenesis studies in cultured mammalian cells at the *HPRT* gene [e.g., see (*39*)] and also in germ cells of mice [e.g., see (*5, 40, 41*)] have consistently indicated that large deletions are the predominant type of mutations induced by radiation.

REASONS FOR THE PAUCITY OF DELETIONS IN THE GENOME

There are at least four hypotheses, which are not mutually exclusive, to explain the difference between expected and observed outcomes.

Hypothesis 1

The results of mouse SLT studies might consist of a large fraction of untargeted dominant mutations, which occurred at nonselected loci or epigenetic mutations at the selected loci.

Hypothesis 2

Radiation exposures can induce mutations more or less equally in the genome but deletion mutations are often large enough to involve essential genes, which leads to negative selection events in mutant cells during the mitotic and meiotic processes in spermatogonia cells and/or embryonic or fetal development after fertilization.

Hypothesis 3

The microarray-CGH method was not sensitive enough to detect small deletions and overlooked many of them.

Hypothesis 4

Radiation response varies extensively among genes. Genes used for the SLT studies were unusually sensitive to radiation while many other genes in the genome would mutate only at low frequencies in response to radiation exposures.

Likelihood of Hypothesis 1

In the mouse SLT studies, each mutant is subjected to allelism tests (*42*, *43*). Specifically, a mutant mouse is mated with mice bearing a balancer chromosome (an

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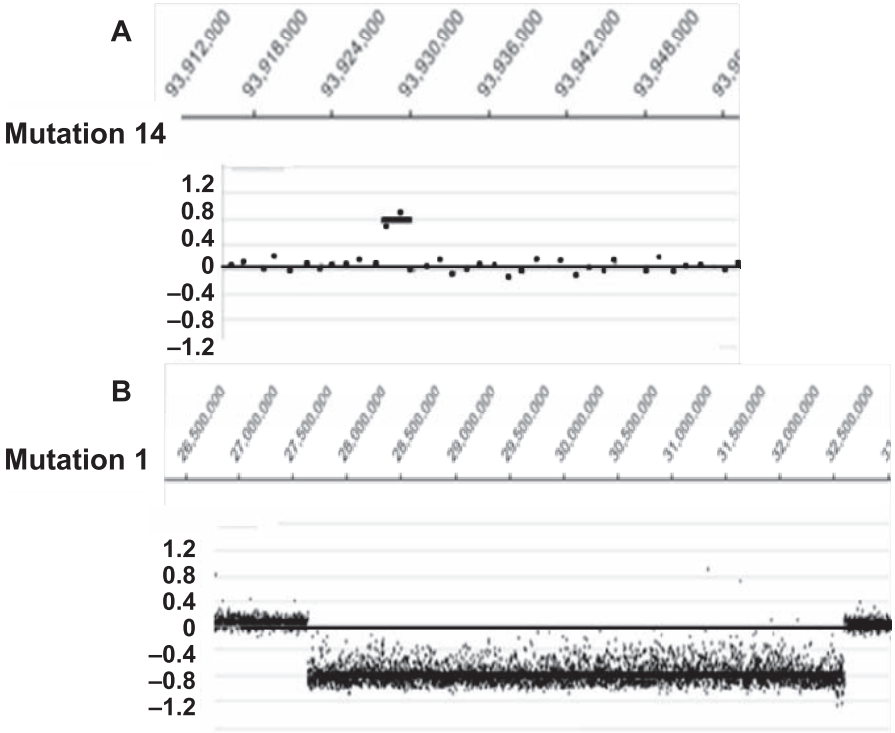

FIG. 3. Two deletions detected using the microarray-CGH method. Panel A: Only two probes were affected here (a 1.7-kb deletion). Panel B: A total of 4,664 probes were affected (a 4,967-kb deletion). In panels A and B, the y-axis represents the ratio (in log scale) of the fluorescence intensities between the green and red colors, and the x-axis represents the magnified area of a chromosome where the deletion was observed. Each dot represents each probe (*34*).

inversion-bearing chromosome) to create mutation heterozygotes ($+^{mut}$/inversion) that will be subsequently used to produce mutation homozygotes to determine if the new mutant allele shows any phenotypic effect different from that of the original tester strain. Under such experimental conditions, if a mutation, which occurred at a non-selected gene, had affected the phenotype of F1 animals born to the irradiated parents, it has to be a dominant mutation and should affect also the F2 individuals ($+^{mut}$/inversion). In other words, such mutations, if they occur at all, are clearly distinguished from recessive mutations that occurred at selected loci. Therefore, this hypothesis cannot explain the paucity of deletion mutations when the whole genome was subjected to the analysis. One might also raise a possibility that epigenetic mutations could have extensively inflated the estimated mutation induction rates per locus. However, a substantial fraction of radiation-induced mutations are known to accompany deletions (*4, 44*, *45*), and thus, epigenetic events, if there are any, cannot be the cause of the large discrepancy between expectations and the observed outcomes.

Likelihood of Hypothesis 2

One may assume that genes, such as those that determine coat colors, are free from deleterious effects on the viability of the animals, and thus the results of the SLT studies could be different from, for example, other genes involved in physiological pathways. However, one mutant allele (d^{l20J}) at the *d* locus (one of the specific locus genes used in the SLT), which was known to cause homozygous lethality, was found to be an intralocus deletion (*41*); also, an artificially knocked-out null allele of the *s* gene (another gene used for SLT studies) caused homozygous lethality (*46*). These results raised the question of why the mutant homozygous animals used for the tester strain were viable and fertile. This question was answered when it was found that the mutant *d* locus allele used for the tester strain was derived from an old mutation found among animals from mouse breeders and collectors, and was caused by the insertion of a viral genome (*47*) and animals were hypomorphic (with low function, not a null function). The same was true for the *s* locus mutant used for the tester strain (*48*). These results indicate that the *d* and *s* genes are not simply coat color genes, but are indispensable, which can be regarded as counter evidence against the hypothesis.

Another observation is that the 2DE method with ^{32}P-labeled DNA is sensitive enough to detect small deletions, while mutation induction rates at CG-rich (gene rich) and AT-rich (gene poor) sequences did not show any differences (*36, 49*). Thus, no evidence was obtained to indicate that many deletions occurring in gene-rich regions were negatively selected. These observations do not support hypothesis 2.

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On the other hand, recent genome-wide knock-out studies using the gene-trap method in haploid cell lines (*50, 51*) or the CRISPR system in human diploid cell lines (*51, 52*) suggested that nearly 2,000 genes are essential for cell growth. If we suppose that all of those genes show haploinsufficiency under heterozygous conditions and are distributed uniformly throughout the genome, the mean distance between the two neighboring genes would be approximately 1,500 kb (1.5 Mb). This size is large enough at the molecular level to induce genetic diseases but not large enough to be detected under the light microscope (G-banding of metaphase chromosomes at the 400-band level will give one band of approximately 10 Mb). Thus, if there are 2,000 genes that may drastically reduce the survivability of heterozygotes, it is possible that many deletion-bearing germ cells may stop dividing or fail to undergo meiotic and developmental processes properly, and ultimately be negatively selected. This possibility may favor hypothesis 2. However, it remains to be known if mutations in those genes severely affect the viability of the cells under heterozygous conditions.

Likelihood of Hypothesis 3

For the validity of this claim, small deletions have to be the predominant type of mutations induced by ionizing radiation in germ cells, which is not in line with past experimental data.

1. For example, 18 spontaneous mutations at the albino gene of mice were all viable under homozygous conditions, whereas among 51 X- or gamma-ray-induced mutations, 30% were homozygous lethal, indicating that radiation-induced mutations often consist of deletions that include a neighboring essential gene(s) (*40*).
2. The 2DE method is unique because the detection efficiency of a deletion is independent from the deletion size. Namely, small deletions move the mutant spots only slightly, which results in formation of doublets consisting of one normal and one mutant allele (the spot intensity of each allele is one-half of that seen in normal spots consisting of two alleles). A good example is the smallest deletion that lost only six bases. On the other hand, a large deletion simply results in the disappearance of the mutant spot and thereby reduces the spot intensity to one-half. Nevertheless, the observed number of deletions with the 2DE method was nearly 1/10 of that expected from the results of the SLT studies (*37*).
3. As for the array-CGH technique, the arrays we used had two-million probes, which represent mean interprobe distance of approximately 1 kb. To screen candidate deletions, an effort was made to collect as many candidates as possible, which consisted of altered green/red ratios at two or more consecutive probes (one-probe alterations are too noisy to screen). A pilot test of 17 known polymorphic small deletions (2–4 kb) affecting 2–4 probes between B6 and C3H genomes showed that they were all detectable under heterozygous conditions. The probability of falsely overlooking two-probe deletions relative to large deletions was estimated at less than 5% (*34*). Subsequent screening of 100 offspring born to 4 Gy irradiated sires and 100 controls showed 278 candidate CNVs that were affected at two adjacent probes, and 28 candidate CNVs at three or more probes. Validation tests with PCR showed there were only two true deletions in the former (2/278) while there were 21 in the latter (21/28). These results are not consistent with the possibility that two-probe deletions were induced with much higher frequencies than ≥3-probe deletions, but were mostly overlooked.

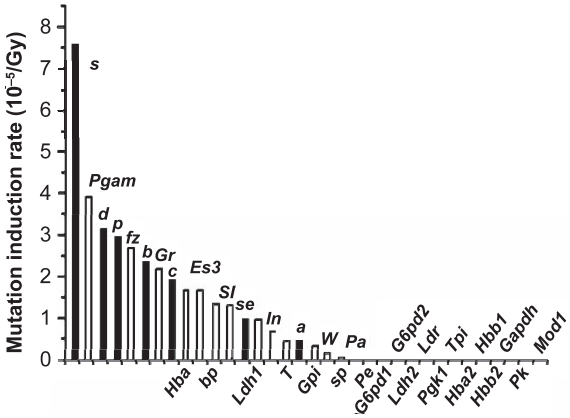

FIG. 4. Mutation induction rate per Gy estimated from mouse specific locus tests (SLT) (*53*). The black bars represent the genes employed in the seven locus tests performed in the U.S.

Likelihood of Hypothesis 4

As shown in Fig. 4, which is a summary of data compiled in an UNSCEAR report (*53*), the SLT studies provided evidence that mutation induction rates varied extensively among different genes. Such observations were also previously made in mutagenesis studies of fruit flies. Figure 4 shows that nearly one-half of the genes tested did not show mutants (but this may be due to small sample sizes), while some genes responded with notably high frequencies.

In addition, from the era of *Drosophila* genetics, it was observed that genes with higher spontaneous mutation rates tend to show higher responses after irradiation (*54*). The trend is also observed in mice [mouse data are also summarized in (*54*)] (Fig. 5). In the past, it was thought that larger gene sizes (target size) may explain the observed intergene differences, however, this was disproved after sequence data for the whole genome were acquired. Specifically, the *a* (non-agouti) and *se* (*Bmp5*) genes are relatively large (260 kb and 124 kb, respectively) but the mutation rates (both spontaneous and radiation-induced) are low. The *s* (*Ednrb*) gene is 20 kb and *b* (*Tyrp1*) is 18 kb, thus both are small genes, but are more mutable than the *a* or *se* genes. As for other gene sizes, *c* (*Tyr*) is 150 kb, *p*

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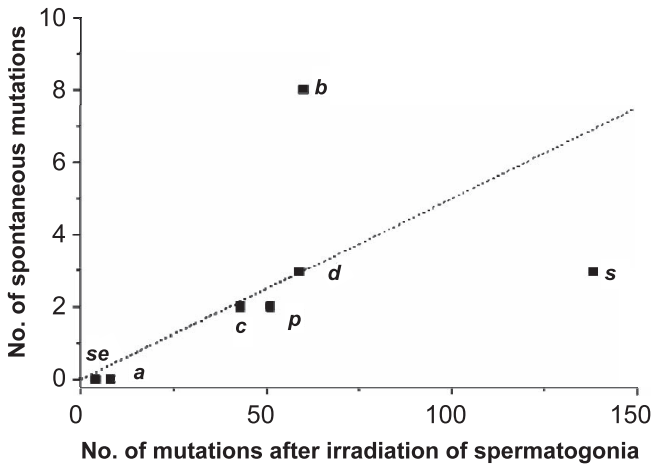

FIG. 5. Mutation rates of the SLT genes used in mice. The graph shows the correlation between the number of spontaneous mutations (y-axis) and mutations after spermatogonia irradiation (x-axis) (*54*).

(*Oca2*) is 296 kb and *d* (*Myo5a*) is 153 kb (the abbreviations in parentheses are the newer or current names of the genes).

Furthermore, recent large-scale human genome data indicate that the spontaneous mutability of genes is highly variable. For example, more than 50% of rare exon variants occur in only 2% of the genes (*55*), and the frequency of *de novo* mutations varies by approximately $\geq$100 times among different genomic regions (*56*). Taken together, it seems that hypotheses 1–3 are less likely when compared to hypothesis 4.

Finally, the induction rate of recessive lethal mutations was once estimated after spermatogonia irradiation and was 0.9% per genome at 1 Gy (*57*). This estimate is surprisingly close to the deletion induction rate of 1% found by the array-CGH method. Because deletions detected with the array-CGH method in the offspring born to irradiated sires are quite large (>200 kb) and include many genes (*58*), the close agreement is understandable if one assumes that at least one vital gene was involved in the deletion.

In short, SLT was an established method used in *Drosophila* genetics, while in mice the mutant alleles studied were obtained from animals that were historically known to be pet mice. Such genes as these might be prone to undergo spontaneous mutations. In terms of evolutionary perspectives, being capable of changing the coat color could be advantageous for survival of the species under sudden environmental changes. Such examples are indeed reported in deer mice (*59*), pocket mice (*60*) and oldfield mice (*61*), and they involved the *agouti* gene, which was one of the seven loci used for the SLT studies.

FUTURE PERSPECTIVES

Unit to Describe Mutagenic Effects of Radiation in Germ Cells

Since it is becoming evident that individual genes behave differently in response to radiation exposure, which might be partly attributable to differences in the intranuclear positioning of genes in each chromosome (*62–64*) and of intrachromosomal gene density (*65*), the classic expression of ''mutation induction rate per locus'' does not seem to be the best way to describe the mutagenic effects of radiation. However, alternative expressions are also not free from problems. For example, ''frequency of deletions per genome'' may appear to be more informative and better than the classic expression, while radiation-induced deletions are generally larger (i.e., many more genes are involved) than spontaneous deletions (*58*); thus, fold increase may not be adequate without considering the general difference in deletion sizes. These problems lead to the idea of ''mean number of genes lost per genome'', but here again, detrimental effects vary among different genes under heterozygous conditions; e.g., a mutant mouse bearing a large deletion of a cluster of olfactory genes did not show any phenotypic abnormality (*58*). Therefore, it is important to understand how often the offspring born to radiation-exposed parents are actually affected. In this respect, the roles of haplosensitive genes may be important.

Haplosensitive Genes and Negative Selection

The above-mentioned problems are derived from the fact that different sizes of deletions are induced in the genome of germ cells after irradiation but only a fraction of them survive and are recovered as live mutants among the offspring. Thus, those deletions that are carried by viable newborns must be generally smaller than those that occurred in lethally mutated zygotes. Possible causes of the lethality associated with deletion mutations are the presence of haploinsufficient (haplosensitive) or haplo-lethal genes. It was in the early 1970s when partial deletions and duplications were produced systematically to cover the whole genome (except for the X chromosome) of *D. melanogaster*. Interestingly, there was a total of 57 haplosensitive genes (heterozygotes are viable but show vulnerable *Minute* phenotypes) but only one haplo-lethal gene (*66*). Thus, large deletions that lead to the death of zygotes in *Drosophila* are interpreted as being due to the additive effects of multiple haplosensitive genes. Compared to the genome of *D. melanogaster*, the genome size of mammals is nearly 10 times larger, while the estimated number of coding genes is less than twice as large (22,000 vs. 15,000). It would be interesting to know if some similar mechanisms that negatively select against deletion-bearing mutants are operating in both species.

There is also the question of how often a haploinsufficient phenotype will occur in organisms. In the mouse, many genes have been knocked out, and the viability and other characteristics have been investigated for both heterozygotes and homozygotes. For example, among 1,700 genes tested, as many as 65% of the genes were found to be nonlethal under homozygous conditions for the mutant allele (probably null allele homozygotes), 11% were sub-viable and 23% were lethal (*67*). Since nearly 80% of the

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genes examined under heterozygous conditions showed at least one abnormal value or trait (including blood biochemistry), the total number of haplosensitive genes should be quite large in mice (note, however, that the definition of a haplosensitive gene differs between fruit flies and mice, as no biochemistry tests were done in flies). However, because the study utilized already established (maintained) lines of mice bearing one knocked-out allele (i.e., heterozygotes) the mutations tested do not appear to profoundly reduce the viability of heterozygotes.

Deletion Polymorphisms in Human Genomes

With the accumulation of individual data for human genome sequences, it became evident that we carry many polymorphic alterations in the genome. Genes involved in polymorphic deletions are unlikely to cause severe developmental diseases under heterozygous conditions, and thus can be called haplosufficient genes. Genes involved in recessive hereditary diseases also belong in this category, since this is the definition of recessiveness. In support of this idea, a large-scale human genome dataset showed that human individuals carry as many as 85–100 inactive genes under heterozygous conditions, and even 20–35 inactive genes under homozygous conditions (*68, 69*). Furthermore, the null function of a gene, the expression of which is restricted to a specific organ(s), may not be recognized as a genetic disease. As observed in mice, paralogous genes may often complement a gene that has lost its function (*68*). In short, our genome is not a solid blueprint, but is functionally flexible, and thus it would be necessary to catalogue the biological effects of each gene under heterozygous conditions in both human genome data and mouse experimental data.

Intergene Differences in Radiation Sensitivity

If the interlocus differences in radiation responses were due to intrinsic differences in the genes and not to an incidental involvement of neighboring essential genes in a deletion, three-dimensional structures of genes can be the key to understanding the difference. For example, some genes may be better protected from radiation damage by the presence of packing proteins associated with the chromatin. The indication that variations in the mutation induction rate among different genes becomes less pronounced after irradiation of post-spermatogonial stages [(*70*); cited in (*45*)] is in line with this concept. To study such a possibility, it would be helpful to understand chromatin structures in different parts of the genome. However, even if such an experimental system could be established in the near future, it would still be a difficult task to apply it to specific genes in germ cells *in vivo*. Alternatively, recent technological innovations have made it possible to culture mouse spermatogonia cells *in vitro*, and to transplant them into the testes of sterile males to reproduce (*71*). Such an experimental system, along with artificial introductions of targeted deletions using the CRISPR system, may open the door to a new and as yet unknown area of genetic research and radiation.

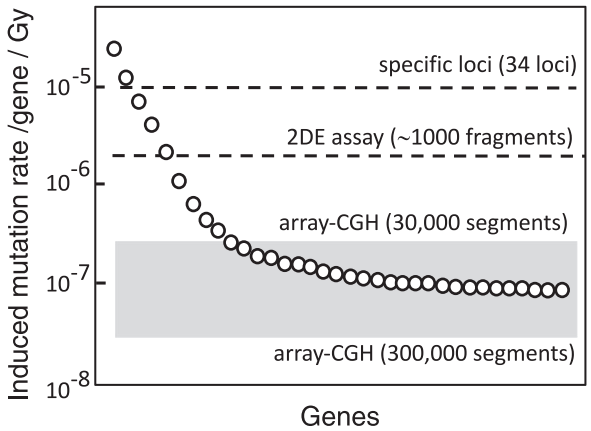

FIG. 6. A hypothetical figure in which genes (x-axis) were lined up according to the mutation induction rate (y-axis) to explain the declining trend of the mean mutation induction rate when the number of genes screened is increased (*34*). The broken horizontal lines indicate the mean frequency of deletion mutations obtained with different methods. The results derived from array-CGH studies are expressed as a range assuming that the genome is made of either 30,000 or 300,000 fragments, each of which consists of a 100 kb- or 10 kb-long stretch, respectively.

CONCLUSION

In the mouse, mutation frequencies in several genes (e.g., genes that involve coat colors) clearly increase in the F1 generation after parental exposure to radiation, however, the induction rate varies largely on the order of 10^{-5} or less/gene per Gy. However, in humans, genes highly responsive to radiation have not yet been found, which forces us to work with the whole genome, while many genes are likely to show only a low mutability. Figure 6 represents a hypothetical scenario in which genes were lined up according to their higher mutation induction rates. This means that without marker genes, which are easily examined and mutate readily after irradiation, it could be difficult to detect the genetic effects of radiation, even in mice. It should be added that any conclusion can change in the future in the light of additional findings through the analyses of whole genome sequences.

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