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Authors: Asakawa, Jun-ichi, Kodaira, Mieko, Miura, Akiko, Tsuji, Takahiro, Nakamoto, Yoshiko, et al.

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Genome-Wide Deletion Screening with the Array CGH Method in Mouse Offspring Derived from Irradiated Spermatogonia Indicates that Mutagenic Responses are Highly Variable among Genes

Jun-ichi Asakawa,[a,1] Mieko Kodaira,[a] Akiko Miura,[a] Takahiro Tsuji,[a] Yoshiko Nakamoto,[a] Masaaki Imanaka,[a] Jun Kitamura,[a] Harry Cullings,[b] Mayumi Nishimura,[c] Yoshiya Shimada[c] and Nori Nakamura[a]

Departments of [a] Genetics and [b] Statistics, Radiation Effects Research Foundation, 5-2 Hijiyama Park, Minami-ku, Hiroshima 732-0815, Japan; and [c] Radiobiology for Children's Health Program, Research Center for Radiation Protection, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan

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Until the end of the 20th century, mouse germ cell data on induced mutation rates, which were collected using classical genetic methods at preselected specific loci, provided the principal basis for estimates of genetic risks from radiation in humans. The work reported on here is an extension of earlier efforts in this area using molecular methods. It focuses on validating the use of array comparative genomic hybridization (array CGH) methods for identifying radiation-induced copy number variants (CNVs) and specifically for DNA deletions. The emphasis on deletions stems from the view that it constitutes the predominant type of radiation-induced genetic damage, which is relevant for estimating genetic risks in humans. In the current study, deletion mutations were screened in the genomes of F1 mice born to unirradiated or 4 Gy irradiated sires at the spermatogonia stage (100 offspring each). The array CGH analysis was performed using a "2M array" with over 2 million probes with a mean interprobe distance of approximately 1 kb. The results provide evidence of five molecularly-confirmed paternally-derived deletions in the irradiated group (5/100) and one in the controls (1/100). These data support a calculation, which estimates that the mutation rate is 1×10^{-2}/Gy per genome for induced deletions; this is much lower than would be expected if one assumes that the specific locus rate of 1×10^{-5}/locus per Gy (at 34 loci) is applicable to other genes in the genome. The low observed rate of induced deletions suggests that the effective number of genes/genomic regions at which recoverable deletions could be induced would be only approximately 1,000. This estimate is far lower than expected from the size of the mouse genome (>20,000 genes). Such a discrepancy between observation and expectation can occur if the genome contains numerous genes that are far less sensitive to radiation-induced deletions, if many deletion-bearing offspring are not viable or if the current method is substandard for detecting small deletions.

INTRODUCTION

Ionizing radiation can induce mutations in both somatic cells and germ cells. In animal experiments, increased frequencies of various types of mutations (e.g., specific locus mutations, dominant lethal mutations, dominant visible mutations, etc.) have been observed in offspring derived from irradiated parents (*1, 2*). In humans, there are two groups of studies: one comprises the offspring of atomic bomb (A-bomb) survivors, and the other comprises the offspring of childhood cancer survivors. In the former study group, because the exposure was derived from a bomb whose primary blast released heat and radiation, many of those who were exposed proximally to the bomb died from blast and burn injuries caused by fire. Consequently, the number of A-bomb survivors who received large doses (e.g., >1 Gy) is relatively small. Among the offspring of A-bomb survivors, there has been no evidence indicating the presence of an excess risk from radiation related to their health (*3, 4*). As for childhood cancer survivors, the patient's gonads have often received repeated exposures to scattered radiation from radiotherapy beams, and the total gonadal dose may reach over 20 Gy. Nonetheless, here too, there is no indication of increased frequencies of malformations, Mendelian diseases or chromosome abnormalities (*5, 6*).

Data from published animal studies is useful in understanding human epidemiologic data. The best known studies are specific locus tests that were performed in the

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[1] Address for correspondence: Department of Genetics, Radiation Effects Research Foundation, 5-2 Hijiyama Park, Minami-ku, Hiroshima, 732-0815, Japan; email: asajun@rerf.or.jp.

1950s and 1960s, primarily at the Oak Ridge National Laboratory (ORNL, Oak Ridge, TN), and to a lesser extent at the Medical Research Council (MRC, Harwell, UK), as well as other laboratories. Specific locus tests used six or seven visible marker genes such as coat color or ear shape (*2, 7*). In these studies, the mean rate of induction of mutations was estimated as 2×10^{-5}/locus per Gy in the 1970s. With more data, and the use of additional loci, the revised rate became approximately 1×10^{-5}/locus per Gy (*2*). However, the individual loci varied widely in their mutational responses [see Table 39 in ref. (*2*)].

We therefore considered it important to design systems that would permit the scoring of mutations in a larger number of loci.

In our earlier published studies (*8–11*), a two-dimensional gel electrophoretic method (2DE or the restriction landmark genome scanning method) was used to analyze DNA fragments labeled with phosphorus-32 (^{32}P) at *Not*I sites (*8*) to screen for deletions under heterozygous conditions at up to approximately 1,000 sites in the genome. With this method, deletions (but not base changes) are detected as spots with 50% decreased intensities in autoradiograms. If the mean rate of induction of the original seven loci were applicable to the rest of the genome, then the expected number of mutations would be $2 \times 10^{-5} \times 1{,}000 = 0.02$ per animal per Gy. This means that using a group of 500 mice should allow the detection of genetic effects resulting from 4 Gy doses (i.e., a net increase of 40 mutations). In our first study, two dose levels, 3 and 5 Gy, were used. A total of only five deletions were found: three in 237 progeny (1.3%) at 3 Gy; one in 79 (1.3%) at 5 Gy; and one in 190 (0.5%) at 0 Gy. In the second study, there was one in 502 progeny (0.2%) in the controls, and five in 505 progeny from the offspring of the 4 Gy irradiated group (1%). The observed frequencies were far lower than expected.

The array CGH method is used for detecting copy number changes (deletions or duplications) in chromosomal segments. Recently, high-density arrays containing over one million probes have become commercially available (each probe is composed of oligonucleotides that are ~60 mers). A ''2M array'' consists of 2.1 million probes with a mean interprobe distance of approximately 1 kb. The number of test sites per genome has now increased by three orders of magnitude when compared with the 1,000 probes used previously with the older 2DE method. Consequently, it was hoped that the array CGH method would be more powerful than the 2DE method in detecting mutations within a relatively small number of F1 animals (e.g., 100 offspring).

MATERIALS AND METHODS

Mice

Two groups of mice were used. The first group consisted of F1 mice derived from crosses of C57BL/6J (B6) males and C3H/HeN (C3H) females (Charles River Laboratories Japan Inc., Kanagawa, Japan). The control series consisted of 100 offspring derived from 15 unirradiated sires (mean: seven offspring per sire). The exposed series consisted of 100 offspring derived from 12 sires irradiated at the spermatogonial stage (mean: eight offspring per sire). The details of individual crosses are shown in Supplementary Table S1 (http://dx.doi.org/10.1667/RR14402.1.S1). When the males reached 10 weeks of age, they were exposed to 4 Gy of ^{137}Cs gamma rays (Gammacell®; Nordion™ Inc., Ottawa, Canada), at a dose rate of 0.5Gy/min, at the National Institute of Radiological Sciences (NIRS; Chiba, Japan). After an 8-week recovery period, the males were individually mated with C3H females to produce 100 offspring (sampling of irradiated spermatogonia).

The second group consisted of 48 offspring sired by B6C3F1 males that were 4 Gy X-ray irradiated and mated eight weeks later to unirradiated JF1 females. This group of mice was previously examined with the 2DE method, which detected three deletions among 237 offspring (*11*), while the 48 mice tested in this report had no deletions detected. This group does not have a matching control group.

When the F1 mice reached approximately 3 weeks of age, the mice and their dams were sacrificed, and spleens, livers and kidneys were removed (the sires were sacrificed when the breeding schedule was over). The isolated tissues were snap-frozen in liquid nitrogen and stored at −80°C until use. All animals were maintained and treated under the Guidelines for the Care and Use of Laboratory Animals. The first group of mice was processed at the NIRS, and the second group at the Radiation Effects Research Foundation.

DNA Samples

High molecular weight genomic DNA was extracted using a proteinase K/phenol extraction method. DNA concentrations were measured using the ultraviolet spectroscopy NanoDrop ND-1000 method (NanoDrop Technologies Inc., Wilmington, DE) and the quality was checked on 0.4% agarose gel. Spleen DNA samples were used for the array CGH experiments while kidney and liver DNA samples were used to confirm candidate mutations detected with spleen DNA. When results from the three DNA samples did not agree, the mutation was regarded as a mosaic mutation and not as a germline mutation.

Microarray Construction

High-density microarrays specific for mouse whole-genome analysis were designed by referring to the mouse reference sequence MM9 (NCBI Build 37) and were synthesized in situ by photolithography on glass slides using a random positional pattern by Roche NimbleGen (Madison, WI). Each probe was synthesized in a square region (13 μm on a side), and 0.7–2.1 million oligo-probes were arranged in a checkerboard pattern. Probes are serially numbered from chromosome 1 to chromosome Y and from centromere to telomere (the Y chromosome was excluded from this study). Probes for the genome, which were from sites adjacent to each other, were located randomly on the slides. In addition, a few thousand probes were arranged as position markers on the slides. Probe information regarding base sequences and locations on the arrays, and the locations of the marker probes on the arrays, are offered as a NimbleGen Design file (NGD file). Chromosomal locations for each probe are provided as a Position file (POS file).

Three types of high-density arrays were used: HX1 arrays (100421_MM9_NO_CGH_HX1, 1-plex array with 2.1 million probes, in which the mean distance between two adjacent probes was approximately 1 kb); UX3 arrays (120316_MM9_WG_CGH_UX3, 3-plex array with 1.4 million probes where the mean interprobe distance was approximately 1.5 kb); and HX3 arrays (090825_MM9_WG_CGH_Hx3, 3-plex array with 0.72 million probes where the mean distance was approximately 3.5 kb).

Specifically, among 100 exposed vs. control pairs of DNA samples in the C3B6 offspring, 80 pairs were examined with the HX1 arrays and the remaining 20 pairs were examined with the UX3 arrays. In this series, a dye-swap method was used with the first 35 tests; i.e., each DNA sample was tested twice with alternative dye colors. For the rest of the samples, no dye swap was used; i.e., these latter tests were single experiments using a single set of dye colors. In the second group (48 offspring sired by B6C3F1 males), 24 pairs of DNA samples were examined with the HX3 arrays with no dye swap.

Preparation of DNA Samples for Array CGH

All CGH experiments were performed according to the NimbleGen Array User's Guide (CGH Analysis). DNA samples from a matched pair of exposed and control F1 mice were labeled with cyanine 3 (Cy3) or cyanine 5 (Cy5), respectively, for the first screening test. In a dye-swap pair format, another array was prepared in which DNA samples were labeled with fluorescent dyes in the reverse order and used for the second test. For this purpose, 1 μg of genomic DNA was subjected to strand-displacement amplifications; e.g., DNA was denatured at 98°C for 2 min, followed by incubation at 37°C for 2 h in the presence of Cy3- or Cy5-labeled random nonamers, Klenow fragments and four dNTP mixtures (all reagents were provided in the Dual-Color NimbleGen Labeling Kit). This process yielded ~50 μg of labeled DNA. After denaturation of the DNA, 34 μg each from the Cy3- and Cy5-labeled samples were mixed at a 1:1 ratio and other specific types of oligonucleotides (Sample Tracking Control and Alignment Oligos; NimbleGen) were added. The mixtures were then applied to the microarrays using a NimbleGen mixing chamber, and were incubated at 42°C for 64–72 h in a NimbleGen Hybridization System 12. In a similar manner, DNA samples labeled with a reversed dye combination were also applied to the second microarrays. After the hybridizations, the arrays were washed in NimbleGen Wash Buffer at 42°C, as indicated in the NimbleGen Array User's Guide, to remove nonhybridized DNA fragments from the slides.

Scanning Strategy for the Arrays

After hybridizations, arrays were washed, dried and scanned. Since Cy5 is known to be sensitive to oxidation from ozone, all procedures after hybridization were performed in a custom-made ozone-free chamber ($80 \times 120 \times 180$ cm). The ambient ozone in the chamber was degraded by forced circulation of chamber air through two chemical filters leading to the destruction of ozone (NT-70: Ozone Solutions, Iowa and HONEYCLE™-ZV: Nichias Co., Tokyo Japan). Ozone concentrations were maintained below 5 ppb. Each array was scanned five consecutive times at a 2 μm resolution with wavelengths at 532 and 635 nm using a MS 200 Microarray Scanner (NimbleGen).

Setting Discriminative Values for Log_2 Ratios

Prior to each measurement, slides were subjected to a set of quality control checks: 1. Visual inspection of the pre- and post-hybridized images at low magnification; and 2. Visual inspection of the scans and pseudo-color plots. Only samples that met quality criteria (i.e., >90% of the cases) were used for downstream data analysis. Also prior to segmentation analysis, qspline fit normalization (*12*) was applied to the data to compensate for inherent differences in signal intensities between the two dyes. To determine the discriminatory $\log_2$ values for selecting CNV candidates, 12 DNA samples that contained previously characterized deletions were used; they consisted of 10 large deletions (360 kb to 13 Mb) and two relatively small deletions (32 and 63 kb). Results from the CGH analysis, using both segMNT and an in-house program written in the statistical language "R" (in-house R program; see Supplementary data; http://dx.doi.org/10.1667/RR14402.1.S1), showed that each deletion was clearly represented by a number of consecutive probes bearing $|\log_2$ ratios$|$ larger than the background variations in normal regions. It was found that using the $|\log_2$ ratio$| > 0.3$ as a discriminative value was effective in selecting deleted regions. The segMNT algorithm identifies DNA segments (or blocks) bearing the same levels of deviations in the $\log_2$ ratios from the baseline (e.g., one copy gain or loss in an autosomal block) and produces the corresponding genomic positions (chromosome number and base pair coordinates), mean $\log_2$ ratios, and the number of probes involved in each block.

Data Processing

Since the data file containing the genomic location and $\log_2$ value for each of the 2.1 million probes is extremely large, probes bearing $|\log_2$ ratios$| > 0.3$ were first sorted out using the in-house R program. The CNV candidates were then selected by screening for clustered alterations in the $\log_2$ ratios at adjacent probes with the aid of serial probe numbers (see below). The data file, termed a general feature format (GFF) file, can also be visualized as a graph of dot plots (each dot represents the $\log_2$ ratio at each probe according to their genomic locations) using SignalMap software (NimbleGen). Brief observations of the dot plots are useful in determining the rough positions of large CNV candidates encompassing a number of probes, or in guessing at the possible structural changes of apparently complex CNV candidates for subsequent PCR confirmation. When a deletion occurred in a male X chromosome, the copy number was expected to change from one to zero, which was detected as a shift of the $\log_2$ ratio from -1 to $-\infty$.

Probes that indicated copy number changes were recorded for each pair of DNA samples, and their chromosomal locations were recorded in a common Microsoft® Excel® file. Alterations that occurred in a single offspring or in multiple offspring from a single pair were regarded as *de novo* CNV candidates, while those that occurred in multiple offspring from different pairs were regarded as candidates for copy number polymorphisms (CNPs), or as indicators of unstable probes. The locations of each CNV candidate from the 100 pairs of mice were used to create a single summary figure (a dot-plot picture) with chromosomal locations so that CNVs seen in different pairs of DNA samples can be seen in a single picture. Each candidate CNV was then examined by comparing the exact locations and relationships of the samples, referring to the genomic locations of altered probes; this is useful for identifying apparently different CNVs as being the same, and thus classifying them as CNPs. CNPs thus observed in multiple individuals born to different pairs were not subjected to further analyses, whereas those seen in single offspring were subjected to a series of validation processes that included ordinary PCR and quantitative PCR (qPCR; see below).

Conditions Used to Minimize False Negatives

In the CGH assays, it is crucial to reduce both the false positive rate (noise) and the false negative rate (which could lead to overlooking CNVs). However, these sets of conditions are contradictory. Ultimately, it is necessary to determine the size of deletions/duplications to screened for. Since it is known empirically that single probe abnormalities are frequently false positives, we tested whether two successive probes could be an effective indicator for abnormalities. For this purpose, a model experiment was performed by using known CNVs in the genomes of the B6 and C3H strains (*13*).

Attention was first focused on 17 regions in the C3H genome that are reported to bear 2–4 kb deletions and also involve 2–4 probes in the array format. Since the B6 genome contained two copies of a region of interest and the C3H genome had none, a 1:1 mixture of B6 and C3H DNA was labeled with Cy3 to mimic deletion heterozygotes, and B6 DNA was labeled with Cy5 to mimic a normal genome. After scanning the array slide five times (but with no dye swap), the mean $\log_2$ ratios of the probes located in each of the 17 deletions was obtained (Supplementary Table S2; http://dx.doi.org/10.1667/RR14402.1.S1). In 16 deletions, at least two adjacent probes within

TABLE 1
Stepwise Decrease in the Number of CNV Candidates after Different Levels of Screening

Screening conditions	Number of affected probes			
	2 probes		≥3 probes	
	U shape	W shape	U shape	Total
In-house algorithms only on individual slides	273	108	252	633
After exclusions of candidates found in multiple offspring[a]	170	108	28	306
After confirmation by qPCR[b]	2	0	28	30
After exclusions of inherited cases[c]	1	0	21	22[d]

[a] Candidates that appeared in multiple offspring were regarded as copy number polymorphisms (CNPs) or the probes were inherently unstable.
[b] Most of the candidates that turned out to be false positives were derived from no-dye swap experiments.
[c] The excluded eight cases here were found in either parent.
[d] Among these, 14 were deletions and eight were duplications.

each deletion were found to show mean $\log_2$ ratios smaller than −0.3. In the remaining single deletion, which apparently involved three probes, the two outer probes gave $\log_2$ ratios smaller than −0.3 but the central probe did not. Thus, the strategy for deletion screening was defined as follows: any clustered alterations of $\log_2$ ratios at two or more consecutive probes was considered as a deletion candidate (a U-shaped deletion), as were two nearby probes interrupted by one (a W-shaped deletion). Under these conditions, all of the 17 deletion heterozygotes could be identified as deletion candidates, indicating that the probability of overlooking deletions is reasonably low. Also examined were 69 regions of the C3H genome reported to contain larger deletions (4–650 kb), which involved 5–462 probes in the microarray design (*13*) and hence were expected to show an even lower false negative rate. Out of 69 such deletions, 68 were detected as deletion heterozygotes (see Supplementary Table S3; http://dx.doi.org/10.1667/RR14402.1.S1). The remaining one deletion bore three affected probes, and the region between each of the probes was interrupted by three normal probes ($V^{-}V^{-}V$; no. 46). Under these conditions, the probability of falsely overlooking or underestimating the rate of small (involving two probes) deletions relative to large deletions is likely to be less than 5% (i.e., 1/86).

In the case of JF1B6C3F1 offspring (the second group with 48 offspring), for rapid screening, the dye-swap procedure was omitted and ≥5 consecutive $|\log_2|$ values >0.3 were used to screen for CNVs.

Dealing with Single-Probe Alterations

Since it was found that most of the small deletions involving two probes were detectable (although there were many false positives), it was necessary to decide whether or not to include single-probe alterations in screening. Fourteen known single-probe deletions in DBA2 genome vs. B6 genome (*14*) were examined and all were correctly detected. However, nearly 100 single-probe deletion candidates were also detected on each slide under these conditions (dye swap and five scans per slide; results not shown). As expected, none were found to be true deletions after PCR tests of 126 randomly selected candidates. Therefore, we did not pursue an investigation of mutations that affected only a single probe.

Quantitative PCR

The copy number of each CNV candidate was examined with real-time qPCR. PCR was performed in 20 μl capillaries with SYBR® Premix Ex Taq® II reagent (TaKaRa Biotechnology, Osaka, Japan) using LightCycler® Instruments (Roche). The analysis was done using three replicates and the mean value was used to estimate the copy number of each candidate region for CNVs. DNA samples prepared from spleen, kidney and liver were used to exclude mosaic mutants. DNA fragments that contained the deletion/amplification were amplified with PCR and the products were sequenced using a PRISM® 310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) to determine the base sequences surrounding the breakpoints and the exact deletion/amplification sizes.

Statistical Methods

Statistical tests and estimates of the risk of acquiring deletion mutations were obtained using the same method as Adewoye *et al.* (*15*) due to the extremely small number of observed events (mutations). That is, the null hypothesis for testing the statistical significance of irradiated vs. control samples was to: 1. Assume as the null hypothesis that both irradiated and control samples had the same distribution of numbers of deletion mutations (Poisson with a mean of 3 mutations in 100 mice); 2. Generate 100,000 sets of data from this assumption; and 3. Calculate the P value of a one-sided test as the fraction of those realizations for which the difference of irradiated – control samples was ≥4. The confidence interval for the estimate was constructed similarly by using simple trial and error to find the rates per 100 mice that gave a result as extreme or more extreme that the observed result in 2.5% of 100,000 simulations.

RESULTS

A total of 127 CGH experiments were performed to test 100 matched pairs of DNA samples from the control and exposed offspring and the 53 parents. However, the quality of hybridization was not satisfactory for eight slides (approximately 7% of the total) and those DNA samples were tested again with new slides.

Dye Swaps and Multiple Scans Improve Reproducibility

During the initial CGH experiments, we found that repeated scans of the same slides reduced the number of small CNV candidates (2-probe CNVs) substantially; specifically, in three sets of pairwise tests, the total number of 2-probe candidate mutations decreased from 158 (with a single scan and no dye swap) to 57 (with five scans and no dye swap). A dye swap was also effective in reducing the number of apparently false positive candidate mutations drastically; the number of 2-probe candidate mutations decreased from 158 (with a single scan and no dye swap) to seven (with a single scan and a dye swap) and to three (with five scans and a dye swap). However, for larger alterations (candidate mutations encompassing ≥3 probes), neither repeated scans nor a dye swap had a substantial effect on the

TABLE 2
Deletion Size, Chromosomal Location, and Parental Origin of the 14 Confirmed Deletions among 22 CNVs shown in Table 1 after CGH Tests of 100 C3B6F1 Mice Born to 4 Gy Irradiated Sires at the Spermatogonia Stage and 100 F1 Mice of Control Group

Dose	Size (kb)	Chromosomal location	Parental origin
4 Gy	4,967	9	Paternal
	2,668	5	Paternal
	735	14	Paternal
	323	9	Paternal
	74.4[a]	2	Undetermined
	65.1[a]	2	Undetermined
	15.6	10	Undetermined
	4.6	1	Paternal
0 Gy	150	3	Paternal
	20.5	1	Maternal
	14.7	2	Maternal
	13.2	16	Maternal
	4.4	8	Maternal
	1.7	2	Maternal

[a] The two deletions were found in one offspring but the parental origins were undetermined (see Supplementary Table S5 and Fig. S2; http://dx.doi.org/10.1667/RR14402.1.S1).

number of candidate mutations (Supplementary Table S4; http://dx.doi.org/10.1667/RR14402.1.S1). Consequently, we decided to scan each slide five times along with a dye swap for the first 35 matched pairs of samples. The remaining 65 test pairs were scanned five times and a dye swap was omitted, which could have increased the number of 2-probe CNV candidate mutations, but this was acceptable since the number of PCR tests needed still remained in a practical range.

Radiation Effects

Using the in-house R-based program, we found 633 candidate mutations (≥2-probe U-shaped or W-shaped candidates) among 100 pairs of F1 animals (Table 1). Among these, 327 were not *de novo* mutants because they were found in multiple F1 individuals born to different pair mates. The remaining 306 candidates were subjected to qPCR analysis, of which 276 were false positives (had two copies). Approximately 60% of these (168/276) occurred in the results from the CGH experiments without a dye swap and had only two contiguous probes affected (U-shape), and the rest (108/276) were from W-shaped alterations. The remaining 30 candidates were all confirmed as deletion- or duplication-type CNVs (Supplementary Table S5; http://dx.doi.org/10.1667/RR14402.1.S1). However, eight of them were further excluded because six were also observed in the parents of the individual mutant offspring and two were mosaic (no mutations were found in corresponding kidney and liver samples). Finally, 22 were found as *de novo* mutations; namely 14 deletions (Table 2) and eight duplications. CGH patterns of the smallest and largest deletions are shown in Fig. 1 and the remaining ones are

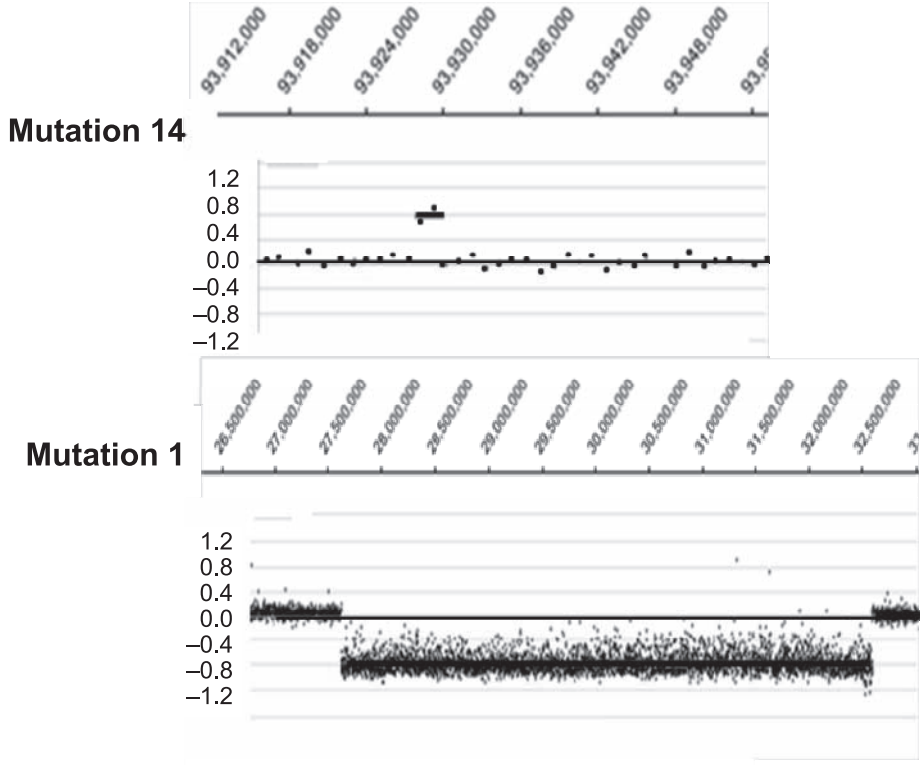

FIG. 1. Two examples of deletion mutations. Mutation 14 has only two probes affected (smallest deletion) whereas mutation 1 has 4,664 probes affected (largest deletion).

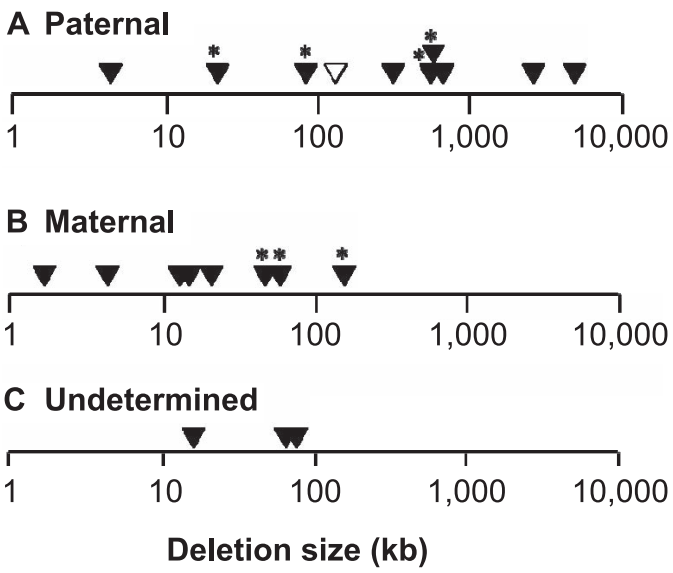

FIG. 2. Distribution of deletion sizes of mutations detected in C3B6F1 mice (n = 100 each in the irradiated and the control group) and JF1B6C3F1 mice (n = 48 in the irradiated group only) (see Tables 2 and 3). Panel A: Deletions in the paternal genome. One open symbol represents a deletion that occurred in the control group, and the rest occurred in irradiated genomes. Panel B: Deletions in nonirradiated maternal genomes. C) Deletions detected in the offspring of 4 Gy irradiated males that were of undetermined origin. *Mutants detected in the 48-mouse study.

shown in Supplementary Fig. S1 (http://dx.doi.org/10.1667/RR14402.1.S1). No cluster mutations (identical mutations among multiple offspring from the same pair mates) were detected in the current study.

The $\log_2$ ratio data were also analyzed with the segMNT algorithm. We found that both the segMNT and in-house R program were equally sensitive in detecting CNVs encompassing $\geq$5 probes. However, for detecting smaller CNVs, the R program detected 460 more candidates than the segMNT method (2–4 probes affected, U- and W-shaped) while only four of them (4/460 or 1%) turned out to be true CNVs (these are CNV nos. 6, 7, 13 and 14, shown in Supplementary Fig. S1; http://dx.doi.org/10.1667/RR14402.1.S1). Unfortunately, these four deletions did not contribute to the estimates of genetic risk because the parental origins of two could not be determined (nos. 6 and 7) and two were maternally derived (nos. 13 and 14). No candidate mutations were identified exclusively by the segMNT algorithm.

Among the 14 deletions, eight were found in the exposed group (five were paternal and three were of undetermined origin) and six in the control group (one paternal and five maternal). For testing statistical significance, analysis was restricted to the six deletions of known paternal origin and the Poisson test was employed, as described by Adewoye *et al.* (*15*) for the five exposed and one control sample. This gives a one-sided *P* value of 0.0737, which can be considered marginally significant.

TABLE 3
Deletion Size, Chromosomal Locations and Parental Origins of Seven Deletion Mutations Detected among 48 Offspring Born to 4 Gy Irradiated Sires

Size (kb)	Chromosomal location	Parental origin
593	3	Paternal
568	13	Paternal
155	10	Maternal
87	8	Paternal
59	X	Maternal
47	1	Maternal
23	10	Paternal

Note. Mutations that affected $\geq$5 consecutive probes only were selectively detected.

Using base sequence information from probes, PCR analysis was performed to amplify the deletion-containing DNA segment so that the precise breakpoint could be identified, and thus the exact deletion size. The results of the deletion sizes are shown in Fig. 2 (information included in Table 2 and Supplementary Table S5; http://dx.doi.org/10.1667/RR14402.1.S1). It varied extensively, from 1.7 kb to approximately 5,000 kb, and small deletions (e.g., smaller than 10 kb) were not common, although three or more probes are likely to locate within a 10 kb span. Therefore, the paucity of small deletion mutations might be a characteristic of radiation-induced deletions occurring in male germline stem cells, although we cannot formally exclude the possibility that conditions for detecting small deletions may be suboptimal in the CGH assay.

In addition to the C3B6 offspring (a total of 200 mice), CGH examinations were also performed on 48 mice born to 4 Gy irradiated B6C3 sires mated with unirradiated JF1 females (JF1B6C3F1 mice). Because small deletions (i.e., $<$10 kb) were relatively rare, the coarser arrays were used here (HX3 arrays with a mean interprobe distance of 3.5 kb) without a dye swap, and abnormal $\log_2$ values at $\geq$5 consecutive probes were used to screen for large CNVs (with a minimum deletion size of $\geq$20 kb). In this way, seven *de novo* deletions were detected, of which four were paternal and three were maternal in origin (Table 3; indicated with asterisks in Fig. 2). It is notable that the deletions that were detected in the irradiated paternal genome were often larger than 100 kb, while those that occurred in the unirradiated maternal genome were generally smaller than 100 kb (Fig. 2).

DISCUSSION

Concordance between Three CGH Studies

In this study we presented data on the induction of CNVs in spermatogonial stem cells from C3B6F1 strain mice where the CNVs were detected using array CGH (2.1 million probes with a mean interprobe distance of approximately 1 kb). After receiving a dose of 4 Gy gamma radiation, a total of 12 CNVs (eight deletions and four

duplications) were found in 100 offspring. Five of the eight deletions could be confirmed as being of paternal origin, and for the remaining deletions, the origin could not be determined. Two of the four duplications were paternal and the other two were maternal. In the controls (n = 100), a total of 10 CNVs (six deletions of which only one was of paternal and four duplications of which two were paternal) were found. The deletion frequencies were therefore composed of 5% in the 4 Gy dose group and 1% in the controls, and the rate of induction of deletions was 1×10^{-2}/Gy per genome (95% CI: ~0.001, 0.028 based on Poisson statistics). In the part of this work involving 4 Gy irradiated B6C3F1 males mated with JF1 females (*11*), four paternally derived deletions were detected among 48 offspring (8.3%) but there were no controls. If the control frequency of 1% for the C3B6F1 strain also applies here, the induction rate becomes: $(8.3 - 1.0) \times 10^{-2}/4 = 1.8 \times 10^{-2}$/Gy per genome. Of note, although the arrays used were coarser (by a factor of approximately 3) than those used in the C3B6F1 study, and the screening conditions were more stringent (i.e., an altered $\log_2$ ratio at $\geq$5 consecutive probes), the observed deletion frequency was not very different from that observed in the C3B6F1 study. These results are understandable because the deletion sizes in the irradiated genome were often larger than several hundred kb (Fig. 2), which is much longer than the minimum deletion size of approximately 20 kb in the JF1C3B6F1 study.

In an independent published study, using array CGH assays similar to those used in the current study, Adewoye *et al.* (*15*) found nine deletions in 100 progeny (9%) derived from mating X-ray irradiated C57BL/6 males (3 Gy; spermatogonial irradiation) to unirradiated CBA/Ca females. After correcting for controls (1/93 or 1.1%), the rate of induction of deletions in this study can be estimated as 2.4×10^{-2}/Gy per genome [based on the pooled data for premeiotic (9/100) and post-meiotic cells (5/69) together (14/169)]. When considering the genomic rates of induced deletions discussed in the preceding paragraph, namely 1×10^{-2}/Gy and 1.8×10^{-2}/Gy, and the small sample sizes, it can be concluded that they are in good agreement with one another. The unweighted average of these estimates is approximately 1.7×10^{-2}/Gy.

Comparisons with Data on Recessive Lethal Mutations

There is one other genomic mutation rate published in the literature: the X-ray induction of autosomal recessive lethals in mice. In the 1960s and 1970s, Lüning *et al.* examined the dynamics of the accumulation of autosomal recessive lethals in irradiated mouse populations (*16–18*), using inbreeding techniques and post-implantation mortality to assess the presence of recessive lethals. The focus of their work was on progeny descended from irradiated spermatogonia. In retrospect, this work can be considered as an early attempt to study radiation-induced deletions in spermatogonia. The authors estimated the rate of induction to be approximately 0.9×10^{-2}/Gy per genome with 95% confidence limits of 0.4×10^{-2} and 1.5×10^{-2}. This rate is similar to the one estimated from the genome-wide induction rate for deletions.

It is instructive to compare these data on genomic rates with those on induced specific locus mutations (the original seven loci used most extensively at ORNL and elsewhere, the six loci used at MRC, and biochemical loci and dominant visible mutations identified in the course of specific locus experiments). Of note, depending on the group of loci used, the average value of the rate per locus is distributed widely, being highest for the original seven specific loci and going down to approximately 10% of this value for some biochemical loci. When all of the 34 loci, for which estimates are available, are considered (Supplementary Fig. S2; http://dx.doi.org/10.1667/RR14402.1.S1), it is clear that the spectrum is dominated by loci at which no mutations were recovered (14 loci). Only one locus (the *s* locus) has a high mutability rate (8×10^{-5}; Supplementary Fig. S2) and the other loci are between this and zero. The unweighted average rate for the 34 loci is approximately 1×10^{-5}/locus per Gy (*2*).

Estimated Number of Radiation-Responsive Genes

If, for the sake of argument, it is assumed that the mutational responses of the specific loci are considered representative of the mouse genome and that all the recovered mutations are deletions, the average-per-locus rate can be used along with the estimated genomic rate to determine the effective number of loci (or more correctly, genomic regions) that yield recoverable induced deletions. To do this, one divides the genomic rate by the per-locus rate, and the quotient is the effective number of loci or genomic regions. The calculations can be illustrated using our data for genomic rates: 1×10^{-2}/Gy and 2.4×10^{-2}/Gy and the per locus rate based on the original seven loci (3.03×10^{-5}/locus) and all of the 34 loci (1×10^{-5}/locus) [see tables 37 and 39, found in ref. (*2*), respectively]. The estimates are: 300, 1,000, 700 and 2,400 genomic regions, which fall nowhere near the estimated number of >20,000 genes (*19*) in the mouse genome! These calculations lend support to our view that the effective numbers of gene/genomic regions at which radiation-induced deletions are recoverable constitute a small proportion of the genome. A schematic representation of this model is shown in Fig. 3.

Possible Reasons for Mutational Heterogeneity among Different Genes

The reason for so much heterogeneity in the mutational radiosensitivity of different genes/genomic regions in the genome is of critical importance in risk estimation. There are at least three possible explanations, which are not mutually exclusive: 1. Deletions are induced but many of them are not recoverable in the F1 generation; 2. Genome structure is heterogeneous and different parts of the genome

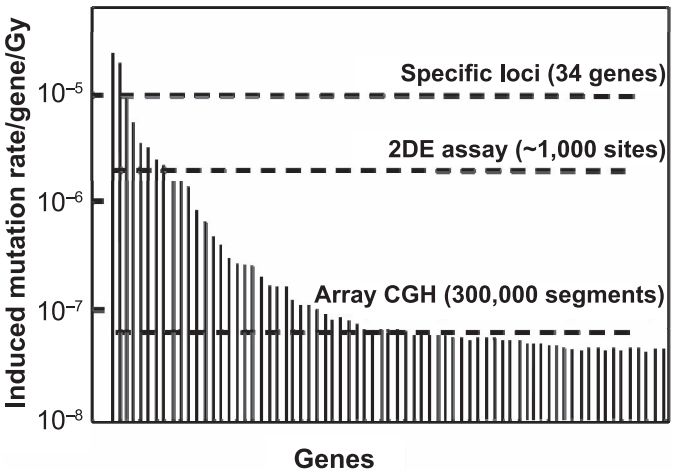

FIG. 3. Schema of the results showing poor radiation response of many genes in a genome. Namely, the mean mutation induction rate per Gy was 1×10^{-5}/locus at 34 loci [specific locus tests (*2*)], 2×10^{-6}/fragment at approximately 1,000 *Not*I sites [2DE method (*11*)] and a 0.6×10^{-7}/10 kb segment of the genome estimated from the array-CGH data (mean mutation induction rate of 1.7×10^{-2}/genome, which consists of 3×10^5 segments that are 10 kb in length).

harbor different sensitivities to mutagenic insults; and 3. It is possible that many small deletions are overlooked in the array studies.

To address the first explanation, Cattanach *et al*. (*20*) provides some clarification through their molecular cytogenetic work on radiation-induced specific locus mutations and some other phenotypes. Their analysis revealed that the distribution of deletions across the genome was nonrandom. For example, they found no deletions in chromosomes 11, 12 and 19, rarely in chromosomes 2, 6, 7 and 9 and multiple examples in some others (chromosomes 1, 3 and 8). Furthermore, deletion sizes were also variable. For example, the *s* locus deletions ranged in size from 2.5 to 30% of the distal region of chromosome 14. Our results also showed multiple deletions in chromosomes 1, 2, 3 and 8 (Tables 2 and 3), but none in chromosomes 11, 12 and 19. These results can be a reflection of the nonrandom distribution of haploinsufficient genes in the genome.

As for the second possible explanation, that the genomic structure is heterogeneous, there are published studies indicating a large heterogeneity among genes for the production of spontaneous mutations; e.g., approximately 60% of rare variants in the protein-coding exome of apparently healthy human populations are found in only 2% of the genes (*21*), and SNPs and CNV losses in germ cells occur more frequently in late replicating DNA (*22, 23*). Furthermore, DNA in isolated nuclei can be protected from radiation injury by compaction (*24*), which suggests that inactive genes may be more protected than active genes from primary injuries caused by radiation. The presence of some type of proportionality between spontaneous and radiation-induced mutation rates (*25*) suggests gene-dependent susceptibility to the two types of mutations. In the field of evolutionary biology, there are arguments suggesting that inherently unstable regions may exist in the genome, which can be reutilized when genome shuffling occurs (*26, 27*). Genes located near such regions may then be more mutable than most other genes located within syntenic blocks.

To address the third possible explanation, that small induced deletions are abundant but mostly overlooked with the array CGH approach, data of whole-genome HiSeq sequencing by Adewoye *et al*. showed a significant, 2.4-fold increase in the induction rate of short indels (<50 bp) (*15*), which may support this idea. However, several lines of evidence suggest it is unlikely that small deletions are induced over ten times more frequently than large deletions. First, in the specific locus tests, homozygous lethality of the mutant allele is a hallmark of large deletions that included a neighboring essential gene(s) and the overall fraction of homozygous-lethal alleles is over 50% (*18*). Furthermore, homozygous-viable alleles also consisted of deletions (approximately 50% of the homozygous-viable *c* locus mutations had deletions of 30–>200 kb) (*28*). Second, among *HPRT* mutants in cultured mammalian cells, the fraction of mutants bearing no detectable alterations using either Southern blots or exon-specific PCR analyses is 50% at most (depending on the radiation dose used), and the fraction of mutants with no detectable alterations decreases with increasing radiation dose (*29, 30*). Finally, in our previously published genome-wide studies using a 2DE method that is capable of detecting losses of a few repeat units in microsatellite sequences, we did not find many small deletions (*9–11*). Nevertheless, technical improvements in genome sequencing technology (deeper and longer reads) could clarify this point in the near future.

In conclusion, our data show that the genomic rate of induced deletions is much lower that what would be expected from the average responses of different sets of specific loci used in radiation mutagenesis experiments. Mouse data on radiation-induced mutations provides the basis for genetic risk estimation in humans, therefore, these findings are clearly relevant. Further investigation is needed to determine whether we have underestimated the number of deletions occurring in the lower size range, or if only a small proportion of the genome is responsive to recoverable induced mutations and to what extent the factors outlined above are involved.

SUPPLEMENTARY INFORMATION

Table S1. Summary of individual crosses with and without radiation exposure.

Table S2. A model experiment to detect 17 known small deletions in the C3H genome.

Table S3. Log_2 ratios of probes located in each of the 69 known deletions in the C3H genome.

Fig. S1. CGH patterns of 22 mutations identified in this study.

Fig. S2. Mutation induction rates at 34 mouse loci [from Table 39 in (*2*)].

Supplementary Data. CGH analysis results were obtained using segMNT and an in-house program written in the statistical language ''R''.

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