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Stage specificity, dose response, and doubling dose for mouse minisatellite germ-line mutation induced by acute radiation

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ABSTRACT **Germ-line mutation induction at mouse minisatellite loci by acute irradiation with x-rays was studied at premeiotic and postmeiotic stages of spermatogenesis. An elevated paternal mutation rate was found after irradiation of premeiotic spermatogonia and stem cells, whereas the frequency of minisatellite mutation after postmeiotic irradiation of spermatids was similar to that in control litters. In contrast, paternal irradiation did not affect the maternal mutation rate. A linear dose–response curve for paternal mutation induced at premeiotic stages was found, with a doubling dose of 0.33 Gy, a value close to those obtained in mice after acute spermatogonia irradiation using other systems for mutation detection. High frequencies of spontaneous and induced mutations at minisatellite loci allow mutation induction to be evaluated at low doses of exposure in very small population samples, which currently makes minisatellite DNA the most powerful tool for monitoring radiation-induced germ-line mutation.**

Several recent studies have shown that ionizing radiation can substantially increase the germ-line mutation rate at mouse tandem repeat minisatellite loci (1–3). We also have studied minisatellite mutation among human families exposed to radioactive fallout after the Chernobyl accident and found a statistically significant increase in mutation rate, most probably caused by radiation (4, 5). The very high rate of spontaneous germ-line mutation at minisatellites means that they are of great value for monitoring germ-line mutation. However, further application of minisatellites as a reliable indicator of the genetic consequences of radiation exposure currently is limited mainly because the mechanisms of induction of minisatellite mutation by ionizing radiation remain unknown, and therefore it is unclear whether induced minisatellite instability is of relevance to mutation induction at other genomic loci. Germ-line mutagenicity studies require a precise understanding of the timing of mutation induction and an evaluation of the dose–response parameters. Our data on minisatellite mutations in post-Chernobyl human families do not clarify either issue (4, 5). Even in mice, three recent studies have produced conflicting results about the efficiency of premeiotic spermatogonia irradiation in inducing minisatellite mutation and have failed to establish any reliable relationship between radiation dose and mutation frequency (1–3). To evaluate mutation induction at minisatellite loci in mice in more detail, we therefore have measured mutation rate after acute paternal exposure to different doses of x-rays and at different stages of spermatogenesis.

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MATERIALS AND METHODS

Mouse Breeding and Irradiation. CBA/H mice (Harwell colony) were used in this study. To obtain control offspring, four nonirradiated males were crossed to eight untreated females (Table 1). Nine males, 8–12 weeks old, were given whole-body acute irradiation of 0.5 Gy (three males) and 1 Gy (six males, including the four used to produce control offspring) x-rays delivered at 0.5 Gy/min (250 kV constant potential, HLV 1.2 mm Cu). Irradiated males were mated to untreated CBA/H females 3, 6, and 10 weeks postirradiation. The animal procedures were carried out under guidelines issued by the Medical Research Council in "Responsibility in the use of animals for medical research" (July 1993) and Home Office project license no. PPL 30/875.

DNA Isolation and Minisatellite Typing. DNA was prepared from tails by using phenol-chloroform extraction (6). Five-microgram samples of DNA were digested to completion with *Alu*I, electrophoresed through a 40 cm long 0.8% agarose gel (SeaKem type LE, FMC) in 1× TBE buffer (89 mM Tris-borate, pH 8.3/2 mM EDTA), transferred to a nylon membrane (Hybond-Nfp, Amersham) and hybridized to ^{32}P-labeled probes as described elsewhere (7). DNA fingerprints were produced by using mouse-specific and human multilocus minisatellite probes MMS10 (8) and 33.15 (9) and two mouse-specific hypervariable single-locus minisatellite probes, Ms6-hm and Hm-2 (10, 11). All autoradiographs were scored over the well-resolved region between 2.5 and 22 kilobases.

Statistical Analysis. Most statistical procedures were described by Sokal and Rohlf (12). The 95% confidence limits for mutation rate and doubling dose were derived from the Poisson distribution (13).

RESULTS

Experimental Design and Mutation Scoring. Two experiments were designed to ascertain mutation induction at different stages of spermatogenesis and to analyze the dose–response relationship for minisatellite germ-line mutation. In the first experiment, the frequency of mutations was measured in three groups of offspring conceived 3, 6, and 10 weeks after paternal 1-Gy x-irradiation. The litters conceived 3 weeks postirradiation were derived from irradiated postmeiotic spermatids, whereas litters from 6 and 10 weeks were derived from irradiated premeiotic A_s spermatogonia and stem cells, respectively (14). In the second experiment, mutation frequencies in nonirradiated families were compared with families derived from males exposed to 0.5 and 1 Gy of x-rays at premeiotic stages (combined data for 6 and 10 weeks postirradiation).

A summary of all mutation data is presented in Table 1. By using two multilocus and two single-locus probes, 30 different

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Table 1. Summary of mutation data

Dose, Gy	Stage, weeks	No. of litters	No. of offspring	Number of mutations scored by different probes: Grand total*	Multilocus probes only: Total	33.15+ MMS10	MMS10 only	33.15 only	Single-locus probes†: Total	χ^2 df = 1‡	*P*-value§	Ms6-hm	Hm-2
0	–	8	54	20 (16)	9	1	7	1	11 (6 + 5)	0.09	0.7679	8 (4 + 4)	3 (2 + 1)
0.5	Spermatogonia (6)	8	45	26 (23)	8	0	8	0	18 (12 + 6)	1.98	0.1590	12 (7 + 5)	6 (5 + 1)
0.5	Stem cells (10)	8	55	41 (36)	19	2	16	1	22 (16 + 6)	4.61	**0.0318**	15 (12 + 3)	7 (4 + 3)
0.5	6 + 10 weeks	16	100	67 (59)	27	2	24	1	40 (28 + 12)	6.50	**0.0108**	27 (19 + 8)	13 (9 + 4)
1	Spermatids (3)	10	62	21 (19)	7	0	7	0	14 (10 + 4)	2.56	0.1093	7 (3 + 4)	7 (7 + 0)
1	Spermatogonia (6)	12	73	60 (53)	20	0	18	2	40 (32 + 8)	15.23	**0.0001**	26 (22 + 4)	14 (10 + 4)
1	Stem cells (10)	5	25	21 (20)	6	0	6	0	15 (12 + 3)	5.60	**0.0180**	7 (5 + 2)	8 (7 + 1)
1	6 + 10 weeks	17	98	81 (73)	26	0	24	2	55 (44 + 11)	21.01	**0.0000**	33 (27 + 6)	22 (17 + 5)
Total		51	314	189	69	3	62	4	120 (88 + 32)	—	—	75 (53 + 22)	45 (35 + 10)
Fraction, %		—	—	—	36.5	1.6	32.8	2.1	63.5	—	—	39.7	23.8

*Number of independent mutations are given in parentheses.
†Numbers of paternal and maternal mutations are given in parentheses.
‡χ^2 test for equal number of paternal and maternal mutations.
§Probability for χ^2 test; statistically significant values are in bold.

minisatellite bands were scored per animal. To determine the extent to which multilocus and single-locus minisatellite probes detect overlapping sets of bands, the same blots were hybridized with all four probes. As previously reported (1), multilocus probe 33.15 also detects bands derived from the highly unstable mouse minisatellites Ms6-hm and Hm-2. Mouse-specific multilocus probe MMS10 detects bands derived from Ms6-hm, some from Hm-2, and additional bands of unknown origin. Mutants scored by all probes were identified as novel DNA fragments present in the offspring that could not be ascribed to either parent. Germ-line mutations at Ms6-hm and Hm-2 were defined as new-length alleles present in offspring showing only two alleles; somatic mosaics with a third nonparental allele (10, 11) have not been included in the analysis.

Most mutant bands (96.3%) were detected by mouse-specific hypervariable probes MMS10, Ms6-hm, and Hm-2 and very few by the human multilocus probe 33.15 alone. The mouse-specific minisatellite probes therefore are very efficient for mutation detection, eliminating the need for human probe 33.15. Establishing the parental origin of mutant bands detected by probes Ms6-hm and Hm-2 was possible because of the extensive multiallelism and heterozygosity seen at these two loci in inbred CBA/H mice. In contrast, DNA fingerprinting of inbred animals with either multilocus probe resulted in relatively homogeneous patterns shared by parents and offspring and does not allow the parental origin of mutants to be determined. Table 1 therefore shows the total number of independent mutations scored by all probes, together with the total number of paternal and maternal mutations scored by the single-locus probes Ms6-hm and Hm-2.

By using the number of mutant bands in offspring, the homogeneity of mutation rate among different treatment groups as well as among families within groups was tested for both experiments (Table 2). This analysis shows that the total and paternal mutation rates differed significantly between stages of spermatogenesis and between radiation doses, whereas the incidence of mutation is homogeneous between families within a treatment group.

Mutation Induction at Different Stages of Spermatogenesis. The frequency of mutation in offspring conceived 3 weeks postirradiation was similar to that in the control group, whereas premeiotic irradiation resulted in a statistically significant increase of mutation rates seen in most families (Table 3, Fig. 1 *a* and *b*). Furthermore, the increase was restricted to paternal mutations after both spermatogonial and stem cell irradiation, consistent with the treatment of males (Table 1). In contrast, paternal irradiation did not affect the maternal mutation rate (Fig. 1 *a* and *b*).

Dose–Response Analysis for Premeiotic Irradiation. Mutation rates scored after premeiotic irradiation at 6 and 10 weeks were similar for litters conceived after 0.5 Gy of paternal irradiation (Fisher's exact test, two-tailed, $P = 0.3674$). The same was true for 1-Gy litters ($P = 0.9999$). We therefore combined data for spermatogonia and stem cell irradiation.

Premeiotic irradiation of 0.5 and 1 Gy caused a statistically significant increase in the paternal mutation rate (Table 3, Fig. 1*c*). Furthermore, an increase was seen in the frequency of paternal mutation with increased exposure, the mutation rate in offspring derived from males exposed to 1 Gy being 1.6 times higher than in families derived from males exposed to 0.5 Gy ($P = 0.0400$, Table 3). The increase was seen in most litters derived from the irradiated males (Fig. 1*d*), and to evaluate the dose–response of mutation induction, we fitted with linear regression the relationship between the number of paternal mutations scored in each offspring, y, and radiation dose ($y = 0.1111 + 0.3379 \times \text{dose}$; $F(1/250) = 15.20$; $P = 0.0001$). The estimates of the regression slope (mutation induction, 0.3379 ± 0.0867 Gy^{-1}) and paternal mutation rate for the control group ($6/54 = 0.1111$ per offspring, Table 1) were used

Table 2. Nested ANOVA analysis for the effect of radiation on minisatellite mutation rate

Experiment, source of variation	df	Total number of mutations: F_s	*P* value	Paternal mutations: F_s	*P* value	Maternal mutations: F_s	*P* value
1 Gy irradiation; 3, 6, and 10 weeks postirradiation (3 groups)							
Between groups	2, 24	15.49	**0.00005**	8.42	**0.0017**	0.57	0.5740
Between families within groups	24, 133	0.49	0.9772	0.66	0.8828	0.89	0.6141
Control and premeiotic irradiation of 0.5 and 1 Gy (3 groups)							
Between groups	2, 38	6.75	**0.0031**	11.31	**0.0001**	0.13	0.8745
Between families within groups	38, 211	0.86	0.7105	0.63	0.9537	0.99	0.5000

F_s and P are the sample statistics of F-distribution and probability, respectively. Statistically significant values are in bold.

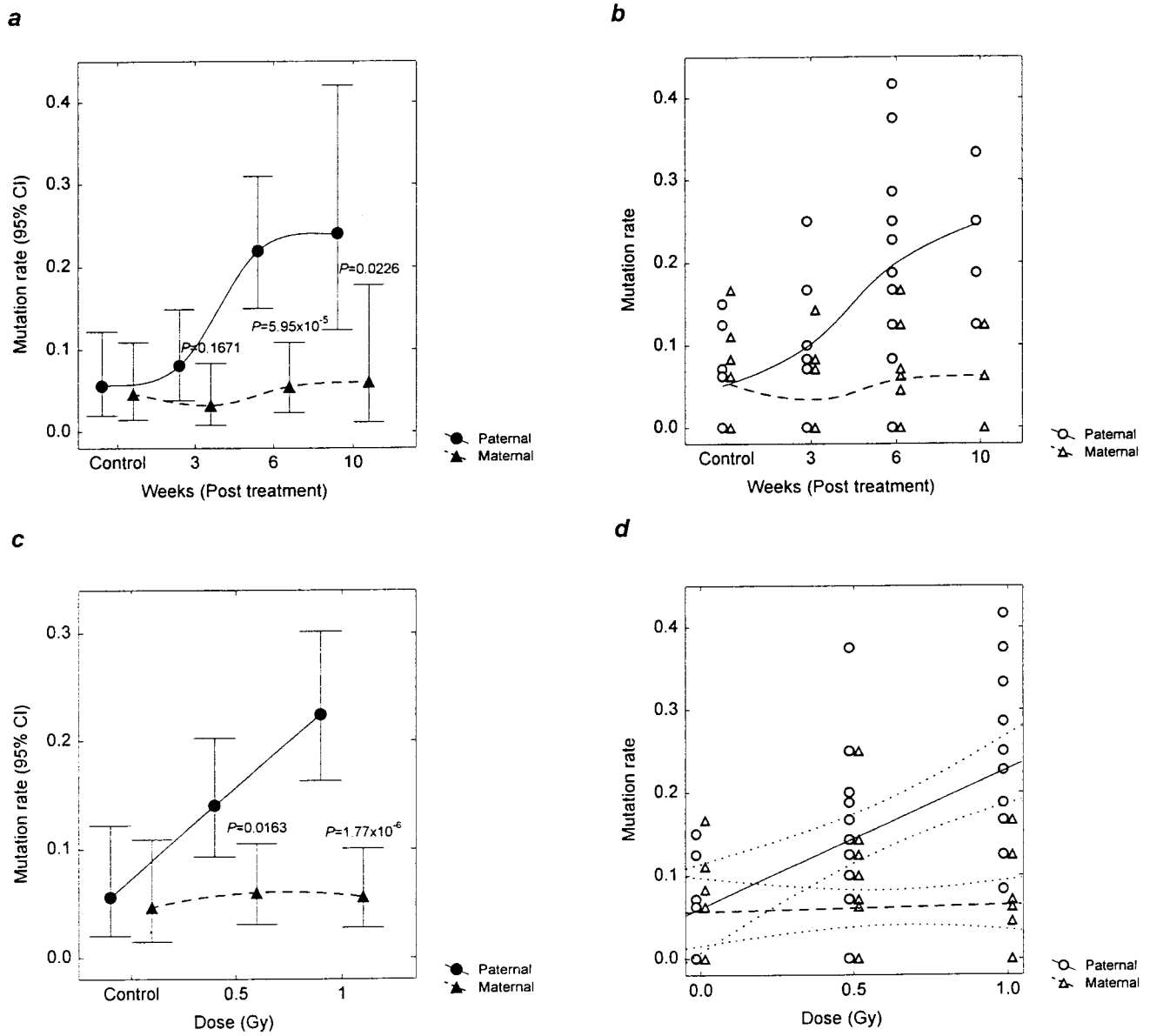

FIG. 1. Mutation induction at minisatellite loci in mice. (*a* and *b*) Frequencies of paternal and maternal mutation in offspring conceived 3, 6, and 10 weeks after paternal irradiation by 1 Gy of x-rays (*a*, aggregated data; *b*, frequency of mutations in each family). (*c* and *d*) Dose–response curves for minisatellite mutations induced in premeiotic cells. (*c*) Aggregated data. (*d*) Frequency of paternal mutation in each family ($r = 0.6131$; $P = 2.0 \times 10^{-5}$ and $r = 0.0888$; $P = 1.0000$ for the arcsine-transformed values of paternal and maternal mutation rates, respectively. Values of Bonferroni probabilities for the coefficients of correlation are given). Dashed lines indicate 95% confidence intervals for the linear regression. The 95% confidence intervals for mutation rate, estimated from the Poisson distribution and probabilities of difference between paternal and maternal rates (Fisher's exact test, two-tailed) are shown for aggregated data.

to estimate the doubling dose for minisatellite mutation as 0.1111/0.3379 = 0.33 Gy (95% confidence interval 0.06–0.75 Gy). In contrast, the frequency of maternal mutation in offspring did not increase with premeiotic irradiation ($r = 0.0185$; Bonferroni probability, $P = 1.0000$; see also Fig. 1 *c* and *d*).

Germ-Line Mosaicism. We previously have shown that mutations at Ms6-hm and Hm-2 occasionally can be shared by more than one offspring, suggesting germ-line mosaicism for a single mutation (10, 11). Only 12% (22/189) of observed mutations could have arisen as the result of germ-line mosaicism, and this frequency did not differ significantly between any of the treatment groups (Table 1). Furthermore, reduction of all instances of shared mutations to a single independent mutation event, while marginally decreasing mutation rates, did not influence the magnitude of mutation rate differences observed between the various treatment groups (data not shown). These data indicate that mutation induction by radiation cannot be attributed to shifts in the level of mosaicism in germ cell populations sharing the same mutation.

DISCUSSION

The results of this study show that the induction of germ-line minisatellite mutations by acute x-rays occurs at the premeiotic stages of mouse spermatogenesis and that the mutation rate increases linearly with radiation dose. These observations are important for the understanding of mutation induction at mouse minisatellite loci and may be of relevance to analogous studies in humans.

The highest radiation dose of 1 Gy was used to compare the induction of minisatellite mutations at three stages of spermatogenesis, revealing that induction is equally efficient for premeiotic stem cell and spermatogonial irradiation, with no

Table 3. Mutation rates in control and exposed groups

Dose, Gy	Stage, weeks	Mutation rate per offspring band								
		Total*	Ratio†	*P*-value‡	Paternal§	Ratio†	*P*-value‡	Maternal§	Ratio†	*P*-value‡
0	—	0.0123	—	—	0.0556	—	—	0.0463	—	—
0.5	6	0.0193	1.56	0.1711	0.1333	2.40	0.0995	0.0667	1.44	0.7510
0.5	10	0.0248	2.01	**0.0129**	0.1455	2.62	**0.0457**	0.0545	1.18	0.9999
0.5	6 + 10	0.0223	1.81	**0.0198**	0.1400	2.52	**0.0325**	0.0600	1.30	0.8280
1	3	0.0113	0.91	0.8930	0.0806	1.45	0.6267	0.0323	0.70	0.8285
1	6	0.0274	2.22	**0.0015**	0.2192	3.94	**0.0003**	0.0548	1.18	0.9978
1	10	0.0280	2.27	**0.0134**	0.2400	4.32	**0.0026**	0.0600	1.30	0.9831
1	6 + 10	0.0276	2.23	**0.0008**	0.2245	4.04	**0.0001**	0.0561	1.21	0.9404

*Total mutation rate was estimated as the number of independent mutations scored in each animal per minisatellite band, assuming 30 bands per offspring.
†Ratio exposed to control.
‡Probability of difference from the control group (Fisher's exact test, two-tailed; statistically significant values are in bold).
§Number of paternal and maternal mutations scored by two single-locus probes, Ms6-hm and Hm-2.

evidence for induction in postmeiotic spermatids. The data also support previous findings that minisatellite loci themselves are not the direct targets of irradiation (2–5). Indeed, if minisatellite mutations found in the offspring of irradiated males are initiated by direct targeted events, this rate would require an unrealistically high number of extra double-strand breaks or other damage per genome. Thus, a 4-fold increase in the paternal mutation rate after premeiotic irradiation of 1 Gy would require 45,000 extra points of damage per haploid genome, assuming that minisatellite loci are random targets (details of estimates are given in ref. 4). In fact, no more than 70 double-strand breaks, 1,000 single-strand breaks, and 2,000 damaged bases are induced per cell per 1 Gy of irradiation (15, 16). If this nontargeted inference is correct, then there are two associated processes leading to radiation-induced minisatellite mutation: structural damage induced elsewhere in the genome or in other sensor molecules and, subsequently, the indirect mutation at minisatellite loci. Our results on apparently similar mutation induction after the spermatogonia (6 weeks) and stem cell (10 weeks) irradiation show that enhanced minisatellite mutation rate can result from damage accumulated in germ cells before meiosis, but does not necessarily indicate that the mutation events themselves occur premeiotically rather than later, for example at meiosis. As the meiotic stages of spermatogenesis have not been included in this study, it is not possible to predict mutation induction during meiosis. Although little is known about the mechanism of spontaneous minisatellite mutation in mice, in humans spontaneous mutation at GC-reach minisatellites is known to be a recombination-based process occurring predominantly in the germ line and most likely at meiosis (17–20). A meiotic origin of induced mutation also would be compatible with our previous results on mutation analysis of human families exposed to chronic radiation after the Chernobyl accident, which showed elevated mutation rates but no evidence of a radiation-induced shift in mutation spectrum or process (4, 5). Long-term chronic exposure of human stem cells could have resulted in considerable accumulation of structural damage to DNA, which may later in some way enhance minisatellite mutation rate at meiosis.

To date, by using several traditional systems for mutation scoring, mutation induction in mice has been compared after premeiotic and postmeiotic irradiation (14, 21). The results of some publications suggest that in male mice A_s-spermatogonia are less sensitive to radiation-induced mutations than postmeiotic spermatids. The pattern of germ cell sensitivity to mutation induction at protein-coding genes is commonly assumed to reflect targeted events and appears to decrease with DNA repair capability in that postmeiotic stages of mouse spermatogenesis are incapable of repair or have a reduced repair capability (21). In contrast, mutation induction at minisatellites reflects nontargeted events and minisatellite mutation itself most probably occurs in diploid germ cells, at least in humans. Thus, in contrast to the protein-coding genes, induction of minisatellite mutation by ionizing radiation may be greatly suppressed after meiosis. However, two recent publications (2, 3) report that the highest frequency of paternal mutation at mouse minisatellite Pc-1 (Ms6-hm) was found after acute irradiation of the postmeiotic spermatids, with no evidence for a statistically significant increase in mutation rate after premeiotic irradiation. In contrast, by using the same minisatellite, we found no sign of mutation induction at the postmeiotic stages, whereas premeiotic irradiation resulted in highly significant 3.7-fold increase in the paternal mutation rate (Table 1, paternal mutation rate 0.0741, 0.0484, and 0.2755 for control, postmeiotic, and premeiotic 1-Gy irradiation, respectively; Fisher's exact test, two-tailed, $P = 0.8458$ and 0.0003 between control, and postmeiotic and premeiotic groups, respectively). The same pattern for mutation induction also was found for another single-locus minisatellite, Hm-2, and for additional mutations scored by multilocus probes MMS10 and 33.15 (Table 1). The results of this study are also consistent with our previous data on mutation induction at mouse minisatellites after spermatogonia irradiation (1). This

Table 4. Estimates of the gonadal doubling dose for acute radiation of spermatogonia in mice

System	Spontaneous mutation rate	Total tested*	Exposure, Gy	Doubling dose, Gy†	Ref.
Russell 7-locus	7.95×10^{-6}	1,051,869	3, 6, 6.7	0.34 (0.22, 0.50)	22, 23
Dominant visibles	8.11×10^{-6}	225,017	6, 12	0.17 (0.00, 0.59)	23
Dominant cataract, 30 loci	7.38×10^{-7}	107,369	1.5-6	0.56 (−0.14, 3.75)	24
Enzyme activity, 12 loci	2.85×10^{-6}	36,422	6	0.44 (−0.09, 2.68)	25
Skeletal malformations	2.88×10^{-4}	2,493	6	0.27 (−0.07, 1.67)	23
Semisterility	1.04×10^{-3}	2,124	12	0.31 (0.03, 0.95)	23
Minisatellites, 2 loci	5.56×10^{-2}	252	0.5, 1	0.33 (0.06, 0.75)	This paper
Mean for 7 systems	—	—	—	0.35 (0.20 0.95)	

*Including offspring from the control and irradiated parents.
†The lower and upper 95% confidence limits computed from the Poisson distribution are given in parentheses.

apparent discrepancy in findings on the timing of mutation induction at mouse minisatellite loci remains unexplained, but we note that previous data have been derived from only one minisatellite locus (2, 3).

We also have analyzed the maternal mutation rate in control families and after paternal irradiation at different stages of spermatogenesis and found no difference in the frequency of maternal mutation. Lack of maternal mutation induction after irradiation of spermatids and spermatogonia also has been shown for mouse minisatellite Ms6-hm (2, 3). In contrast, two publications (2, 3) report a small, but statistically insignificant, increase in maternal mutation rate among litters derived from irradiated spermatozoa. This stage of spermatogenesis has not been included in our experiments.

The second experiment provided evidence for a linear dose–response curve for paternal minisatellite mutation after premeiotic irradiation. These results enable us to compare the sensitivity of different genetic systems in mice to acute premeiotic spermatogonia irradiation (Table 4). We have included in the analysis six different mouse systems for which at least one mutation was found among the control litters, for which the intense selection against *de novo* mutations has not been reported, and where results have been replicated either in the same laboratory or between different laboratories. Here we re-estimate the doubling dose for these six systems with 95% confidence intervals. The results of previous studies appear to be reasonably homogeneous with the doubling dose ranging from 0.17 to 0.56 Gy (Table 4). Most important, our doubling dose estimate for paternal minisatellite mutation, 0.33 Gy, is close to those obtained by using traditional mutation scoring systems in mice, including the specific locus method (Russell 7-locus test), previously the most efficient system of studying point mutations in mice (14). For all seven different monitoring systems, the mean value for the gonadal doubling dose for acute spermatogonia irradiation in mice is 0.35 Gy (95% confidence interval 0.20–0.95 Gy). However, our experiments differ substantially from other studies in the number of offspring scored and the level of radiation used. Statistically significant evidence for mutation induction at minisatellite loci was obtained by profiling only 252 offspring, whereas other systems required the analysis of thousands or even hundreds of thousands of mice to detect significant increases in mutation rate. The resulting profound negative correlation between spontaneous mutation rate and number of offspring analyzed (Table 4) highlights the unique advantage of minisatellite loci for detection of radiation-induced mutations in mice and humans (1, 4, 5). Furthermore, in all previous studies male mice were exposed to very high doses of acute irradiation, far greater than the doubling dose and requiring estimation of the doubling dose by extrapolation assuming a linear response. In contrast, high frequencies of spontaneous and induced mutations at minisatellite loci permitted us to evaluate mutation induction at much low doses of exposure, allowing more robust estimation of the doubling dose without major extrapolation from high doses of radiation.

Analysis of mutation induction at mouse minisatellite loci provides the basis for the possible application of this system for monitoring germ-line mutation in other mammals. The ability of minisatellites to reflect radiation-induced structural damage in DNA induced at the premeiotic stages of spermatogenesis makes them a useful marker for long-term chronic radiation exposure, similar to that after the Chernobyl disaster. Furthermore, acute radiation results in a linear increase in minisatellite mutation rate detectable at doses substantially lower than can be monitored by standard genetic techniques in mice, though the doubling dose is close to estimates previously obtained by using other systems of mutation detection in mice. If correct, then an elevated minisatellite mutation rate should proportionately reflect DNA damage induced by environmental factors, including ionizing radiation. In sharp contrast to previously used genetic systems, this increase can be detected in very small population samples, which currently makes minisatellite DNA the most powerful tool for monitoring radiation-induced mutation in human and other mammalian populations.

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