

Elevated mutation rates in the germ line of first- and second-generation offspring of irradiated male mice

Ruth Barber*, Mark A. Plumb*, Emma Boulton†, Isabelle Roux*, and Yuri E. Dubrova**

*Department of Genetics, University of Leicester, Leicester LE1 7RH, United Kingdom; and †Medical Research Council Radiation and Genome Stability Unit, Harwell, Oxon OX11 0RD, United Kingdom

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Mutation rates at two expanded simple tandem repeat loci were studied in the germ line of first- and second-generation offspring of inbred male CBA/H, C57BL/6, and BALB/c mice exposed to either high linear energy transfer fission neutrons or low linear energy transfer x-rays. Paternal CBA/H exposure to either x-rays or fission neutrons resulted in increased mutation rates in the germ line of two subsequent generations. Comparable transgenerational effects were observed also in neutron-irradiated C57BL/6 and x-irradiated BALB/c mice. The levels of spontaneous mutation rates and radiation-induced transgenerational instability varied between strains (BALB/c>CBA/H>C57BL/6). Pre- and postmeiotic paternal exposure resulted in similar increases in mutation rate in the germ line of both generations of CBA/H mice, which together with our previous results suggests that radiation-induced expanded simple tandem repeat instability is manifested in diploid cells after fertilization. The remarkable finding that radiation-induced germ-line instability persists for at least two generations raises important issues of risk evaluation in humans.

One of the major challenges of modern genetics is to apply recent advances in mutation research to improve the accuracy of the estimates of genetic risks for humans. For example, it has been recognized recently that ionizing radiation not only increases mutation rates in the exposed somatic cells but also results in an elevated mutation rate many cell divisions after the initial irradiation damage (1). In principle, this radiation-induced genomic instability will contribute to the accumulation of oncogenic mutations in somatic cells and malignant transformations (2). If genomic instability is induced also in the germ line of exposed parents, then delayed transgenerational effects may be manifested in their offspring, therefore presenting greater delayed risk in human populations exposed to ionizing radiation.

We recently obtained the first experimental evidence that germ-line mutation rates in unexposed offspring of irradiated male mice do not return to the mutation rates seen in unexposed individuals but are maintained at levels similar to that of directly exposed males (3). These data were generated by using our approach for monitoring germ-line mutation in mice based on a set of hypervariable expanded simple tandem repeat (ESTR) DNA loci (4–7). Unstable ESTRs consist of homogenous arrays of short tandem repeats and show very high rates of spontaneous and radiation-induced germ-line mutations, observed as size changes in the alleles of these loci (4–10). Here we use ESTR loci to study the effects of high- and low-linear energy transfer (LET) exposure and sex and strain specificity on germ-line mutation rates in the first- and second-generation offspring of irradiated male mice.

Materials and Methods

Mouse Breeding and Irradiation. CBA/H, C57BL/6, and BALB/c inbred strains of mice from Harwell colonies were used in this study. Five CBA/H and three C57BL/6 males were given whole-body chronic irradiation of 0.4 Gy of fission neutrons (absorbed dose) using a ^{252}Cf source with a dose-rate of 0.003 Gy·min⁻¹. Seven CBA/H males and five BALB/c males were given whole-body acute irradiation of 2 and 1 Gy of x-rays,

respectively (0.5 Gy·min⁻¹, 250-kV constant potential, half-value layer 1.2 mm Cu). All CBA/H males subsequently were mated to untreated CBA/H females 3 and 6 weeks postirradiation; C57BL and BALB/c males were mated 6 weeks after exposure to control females from the same inbred strain to produce F₁ offspring. F₂ and F₃ offspring were produced from the random mating of male and female F₁ and F₂ mice with control partners (Fig. 1). To ensure the random assignment of F₁ and F₂ parents, all genotyping was performed after the end of the three-generational breeding scheme. All animal procedures were carried out under guidance issued by the Medical Research Council and Home Office project.

DNA Isolation and ESTR Typing. Genomic DNA was extracted from tails by using a standard phenol-chloroform technique. DNA profiles were produced by using two mouse-specific hypervariable single-locus ESTR probes, Ms6-hm and Hm-2, as described (5). Germ-line mutations at *Ms6-hm* and *Hm-2* were defined as new-length alleles present in offspring; somatic mosaics with a third nonparental allele have not been included in the analysis.

Sequencing of *Cdkn2a* and *Prkdc* Genes. PCR primers described in previous publications (11, 12) were used to identify functionally relevant polymorphisms within the genes encoding the proteins p16^{INK4a} (*Cdkn2a*) and DNA-dependant protein kinase catalytic subunit (*Prkdc*). Two intronic regions of each of these genes containing the polymorphisms were PCR-amplified by using 0.2 mM dNTPs, 1 μM of primers, 25 ng of template DNA, 0.5 units of *Taq* polymerase (ABgene, Epsom, UK), 1× PCR buffer (ABgene), and 1.5 mM MgCl₂. Amplification was performed in a total volume of 20 μl in thin-walled 96-well plates on an MJ DNA Engine PTC 220. After initial denaturation at 94°C for 5 min, PCRs were cycled at 94°C for 30 sec, 58–60°C for 30 sec, and 72°C for 1 min for 28 cycles, and ended with a 10-min incubation at 72°C. PCR products were cleaned by electroelution before sequencing by using an ABI PRISM BigDye Terminator cycle-sequencing ready-reaction kit. Reactions were run on an ABI 377 automated sequencer and analyzed by using FACTURA and AUTOASSEMBLER packages (all supplied by Perkin-Elmer/Applied Biosystems).

Results

Experimental Design. The frequency of ESTR mutation was established in the F₁, F₂, and F₃ offspring of irradiated males, which yielded germ-line mutation rates for the F₀, F₁, and F₂ generations, respectively (Fig. 1). The number of mutations scored in each of the three subsequent generations was divided by the total number of offspring in that generation to give an estimate of the parental mutation rate (Table 1). Three exper-

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Abbreviations: ESTR, expanded simple tandem repeat loci; LET, linear energy transfer.

†To whom reprint requests should be addressed. E-mail: yed2@le.ac.uk.

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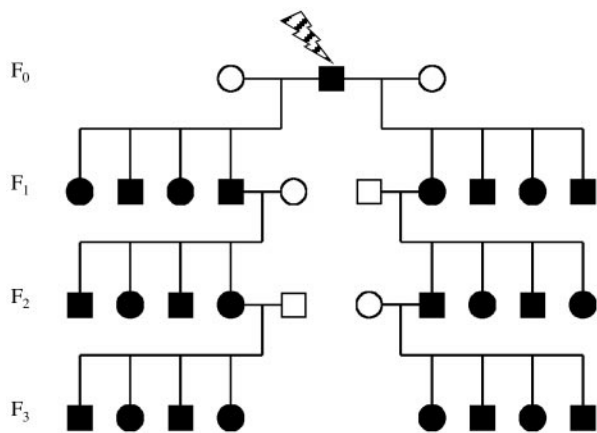


Fig. 1. Design of the transgenerational study. The exposed male and its offspring are in black; control parents with no history of irradiation are in white.

iments were designed. (i) To compare the transgenerational effects of high- and low-LET irradiations, mutation rates were measured in the germ line of offspring of CBA/H male mice exposed to either 0.4 Gy of high-LET fission neutrons or 2 Gy of low-LET x-rays. (ii) To evaluate the transgenerational effects of exposure of post- and premeiotic spermatogenic cells, mutation rates were established in the germ line of first-generation CBA/H offspring and their descendants conceived 3 and 6 weeks after the initial paternal exposure to neutrons and x-rays. The litters conceived during week 3 were derived from irradiated postmeiotic spermatids, whereas those conceived on week 6 were derived from irradiated premeiotic A_s spermatogonia (13). (iii) To compare the transgenerational effects between three

inbred mouse strains, F_1 and F_2 germ-line mutation rates were compared between CBA/H, C57BL/6, and BALB/c mice. These strains have been shown to differ significantly in their radiosensitivity (14) and radiation-induced instability in somatic cells (15–17).

Mutation Induction in the Exposed F_0 Males. Premeiotic exposure of spermatogonia (6 weeks) to low-LET x-rays and high-LET fission neutrons resulted in elevated mutation rate in the germ line of all exposed males (Table 1). In contrast, postmeiotic exposure of spermatids (3 weeks) did not increase ESTR mutation rate in CBA/H males. Neither pre- nor postmeiotic irradiation affected the mutation rate in nonexposed F_0 females (Fig. 2). These data therefore confirm our previous results on the stage specificity of mutation induction at mouse ESTR loci (5, 7).

Germ-Line Instability in Male and Female Germ Lines. Because all inbred strains showed multiallelism and heterozygosity at the *Ms6-hm* and *Hm-2* loci, it was possible to establish the parental origin of mutant bands. The paternal and maternal mutation rates did not differ for the control parents (Fig. 2). Within each strain, male and female offspring from either the first (F_1) or second (F_2) generations born from irradiated F_0 males showed similarly elevated mutation rates in their germ line. The elevated mutation rates observed in the germ line of F_1 and F_2 offspring of irradiated males is elevated significantly, and there is no difference in the transmission of instability through the male or female germ line. It therefore was possible to combine data across the sexes to produce single estimates for the control groups and the F_1/F_2 offspring of irradiated males (Table 1).

Table 1. Mutation rates in the germ line of controls and offspring of irradiated males

Strain, group*	No. of offspring	No. of mutations [†]	Mutation rate	Ratio to control	Prob. [‡]
CBA/H					
Control (8♂, 8♀)	76	22 (20)	0.072	—	—
Neutrons, 0.4 Gy					
F_0 , 3 weeks (5♂)	18	1 (1)	0.028	0.4	0.55
F_0 , 6 weeks (5♂)	43	18 (16)	0.209	2.9	0.001
F_1 (7♂, 9♀)	83	42 (30)	0.253	3.5	2.1×10^{-7}
F_2 (9♂, 7♀)	84	33 (25)	0.196	2.7	0.0002
X-rays, 2 Gy					
F_0 , 3 weeks (7♂)	18	4 (2)	0.111	1.5	0.58
F_0 , 6 weeks (7♂)	47	18 (16)	0.192	2.6	0.003
F_1 (13♂, 8♀)	106	47 (39)	0.222	3.1	2.1×10^{-6}
F_2 (13♂, 11♀)	157	75 (57)	0.239	3.3	1.2×10^{-8}
C57BL/6					
Control (4♂, 4♀)	98	25 (21)	0.064	—	—
Neutrons, 0.4 Gy					
F_0 , 6 weeks (3♂)	45	24 (22)	0.267	4.2	5.5×10^{-7}
F_1 (3♂, 5♀)	58	18 (18)	0.155	2.4	0.006
F_2 (5♂, 5♀)	63	19 (19)	0.151	2.4	0.006
BALBc					
Control (11♂, 12♀)	94	45 (43)	0.120	—	—
X-rays, 1 Gy					
F_0 , 6 weeks (5♂)	32	18 (16)	0.281	2.4	0.003
F_1 (5♂, 10♀)	68	42 (36)	0.309	2.6	2.7×10^{-6}
F_2 (8♂, 6♀)	86	43 (35)	0.250	2.1	0.0003

*The number of male and female parents is given in parentheses.

[†]The number of singleton mutations is given in parentheses.

[‡]Prob., probability of difference from the control group (Fisher's exact test, two-tailed).

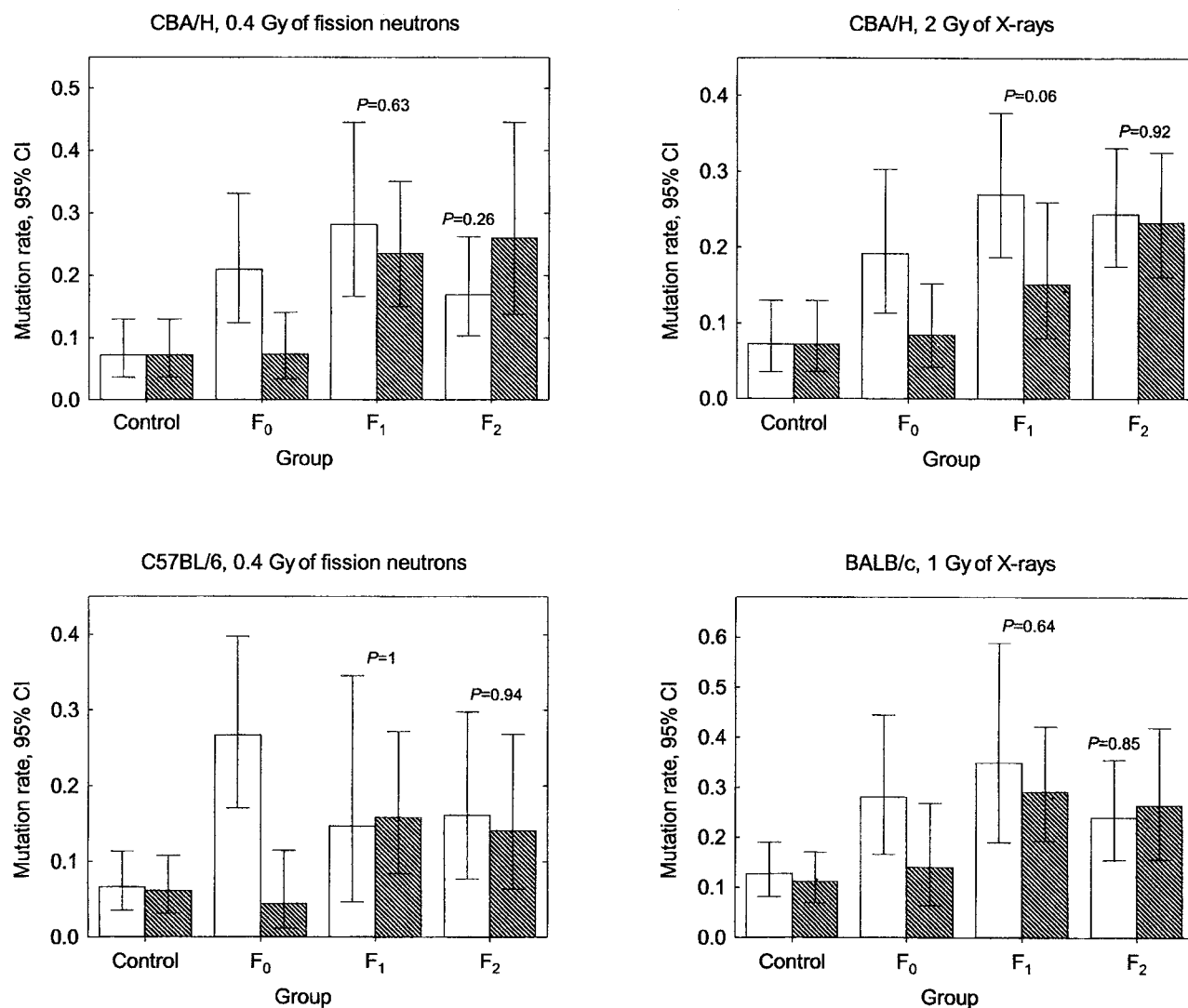


Fig. 2. ESTR mutation rates in the germ line of male (open boxes) and female (hatched boxes) parents. The dates are given for controls, F₀-exposed males, nonexposed females, and F₁/F₂ offspring of irradiated males. The 95% confidence intervals (CIs) for mutation rate estimated from the Poisson distribution and the probabilities of difference between male and female mutation rates in the F₁ and F₂ parents (Fisher's exact test, two-sided) within each generation are shown. The F₀ data for premeiotic exposure (6 weeks after irradiation) are shown.

Effects of High- and Low-LET Exposure. All offspring of CBA/H male mice exposed to either 0.4 Gy of high-LET fission neutrons or 2 Gy of low-LET x-rays showed significant increases in germ-line mutation rates (Table 1). The magnitude of transgenerational increase for these two doses of high- and low-LET irradiations was similar, suggesting that they both can induce germ-line instability in the F₁/F₂ offspring of irradiated males (Fig. 3a). The data for both types of exposure therefore were combined for further analyses.

Stage Specificity for Transgenerational Instability. We have compared mutation rates in the germ line of F₁ offspring of CBA/H male mice conceived 3 and 6 weeks after exposure to fission neutrons and x-rays (Fig. 3b). The magnitude of transgenerational increases for both postmeiotic (3 weeks) and premeiotic (6 weeks) exposures was similar, and mutation rates for the second-generation offspring of pre- and postmeiotically exposed grandfathers also were similar.

Strain Specificity for Transgenerational Instability. The combined data for the CBA/H mice germ-line mutation rates were com-

pared with those for the C57BL/6 and BALB/c strains (Table 1 and Fig. 3c). An increase in germ-line mutation rates was observed in the F₁ and F₂ offspring of all inbred mice; however, the magnitude of both spontaneous mutation rates and transgenerational instability clearly varied between strains. The highest and lowest mutation rates were detected in BALB/c and C57BL/6 mice strains respectively.

By using direct sequencing, we screened the BALB/c, CBA/H, and C57BL/6 mice for the allelic variants of the *Cdkn2a* and *Prkdc* genes previously reported in the BALB/c mice (11, 12). The Harwell BALB/c colony contains a C → A transversion at base 134 (His → Pro) and a G → A transition at base 232 (Val → Ile) within the *Cdkn2a* gene. Also, two changes were confirmed in the *Prkdc* gene: a C → T transition at base 6,418 (Arg → Cys) and an A → G transition at base 11,530 (Met → Val). These polymorphisms were not found in the CBA/H and C57BL/6 mice and are exactly as reported (11, 12).

Transgenerational Instability in the F₁ and F₂ Parents. The frequency of ESTR mutation was established in the F₂ and F₃ litters, which provided estimates of mutation rates for the germ line of F₁ and

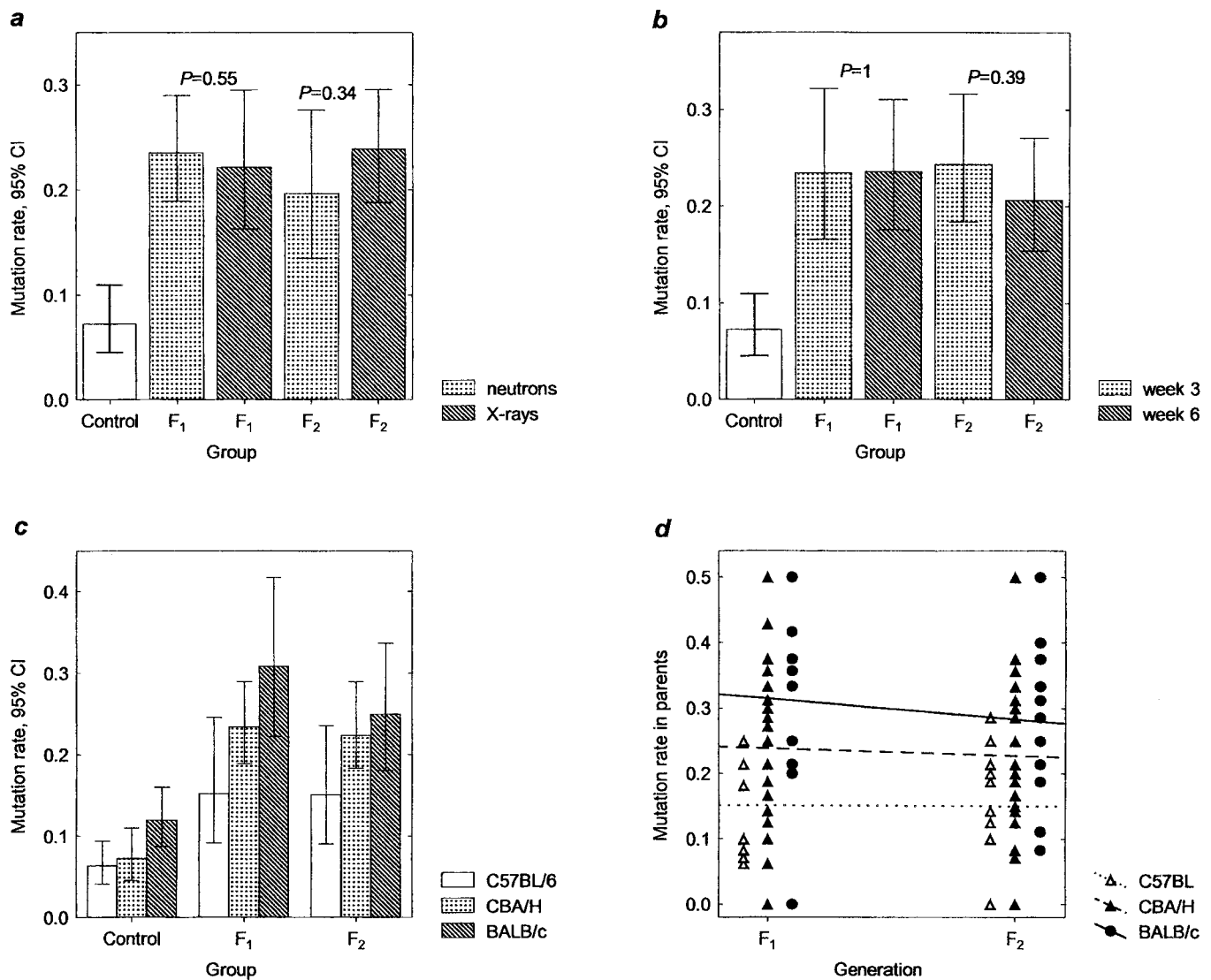


Fig. 3. Transgenerational increases in ESTR germ-line mutation rate. (a) Mutation rates in the germ line of F₁ and F₂ offspring CBA/H male mice exposed to either 0.4 Gy of fission neutrons or 2 Gy of x-rays. (b) Mutation rates in the germ line of F₁ offspring of CBA/H male mice conceived 3 and 6 weeks after paternal exposure to ionizing radiation and in the germ line of pre- and postmeiotically exposed grandfathers (F₂). Aggregated data for the offspring of male mice exposed to x-rays and fission neutrons are given. The probabilities of difference between mutation rates within each generation are shown (a and b). (c) Comparison of spontaneous mutation rates and transgenerational effects in the germ line of three inbred strains of mice. (d) Individual mutation rates in the germ line of F₁ and F₂ parents of exposed males.

F₂ individuals (Fig. 3d). Most F₁ and F₂ parents showed an elevated mutation rate, which resulted in a relatively homogeneous distribution of rates within each strain (χ^2 test for homogeneity of the Poisson distribution, $P > 0.95$, data not shown). By using the two-way ANOVA, the homogeneity of mutation rate (arc-transformed) for all offspring of irradiated parents also was tested and showed that whereas mutation rates differed significantly between strains ($F = 6.97$, $df = 2,118$, $P = 0.001$), they remained similar between generations within a strain ($F = 0.61$, $df = 1,118$, $P = 0.4350$; interaction $F = 0.01$, $df = 2,118$, $P = 0.99$). Thus, within a strain there is no decrease in ESTR germ-line mutation rate from the first to the second generation.

Parental Origin of Alleles and Mutation in the F₁ Generation. Each F₁ offspring contained alleles derived from the irradiated F₀ father and nonirradiated F₀ mother (Fig. 1). In most cases it was possible to establish whether ESTR mutation, occurring in the F₁ generation and detected in the F₂ offspring, happened in the

allele derived from the irradiated F₀ father or nonirradiated F₀ mother. Among 149 *de novo* mutants transmitted from the F₁ parents to their offspring, 63 were traced clearly to the allele derived from the irradiated F₀ father, and 53 were derived from the nonirradiated F₀ mother. The origin of the remaining 35 mutants could not be established unambiguously. We conclude that ESTR instability in the F₁ germ line affects alleles derived from both irradiated and nonirradiated F₀ parents.

ESTR mutation rates in the germ line of the two groups of F₁ parents both with and without mutations derived from irradiated fathers were compared. One-way ANOVA analysis showed that at both ESTR loci, mutation rates in the germ line of F₁ parents containing mutations transmitted from the irradiated fathers were similar to those without paternal mutations (*Ms6-hm*: $F = 0.85$, $df = 1,57$, $P = 0.36$; *Hm-2*: $F = 0.11$, $df = 1,57$, $P = 0.74$). We therefore conclude that the presence of mutations transmitted from the irradiated fathers could not affect the ESTR stability of F₁ parents.

Discussion

The analysis of ESTR mutation in the germ line of first- and second-generation offspring of irradiated males has revealed that (i) germ-line mutation rates were elevated significantly in both generations of all inbred strains studied; (ii) no differences could be observed in the transmission of instability through the male or female germ line; (iii) exposure to 2-Gy x-rays and 0.4 Gy of fission neutrons resulted in similar increases in both generations of CBA/H mice; (iv) the extent of transgenerational increase clearly varied with the different strains; and (v) germ-line mutation rates were elevated equally in the F₁ and F₂ offspring.

To date, only a limited number of studies have addressed the issue of transgenerational changes in the mammalian germ line (18–20). They have shown elevated rates of mortality and malformation in the grand-offspring of exposed males providing indirect evidence for an elevated mutation rate within the first generation. In contrast, the results from our pilot study provide direct experimental evidence for an elevated germ-line mutation rate in the first-generation offspring of neutron-irradiated male mice (3), and we have confirmed this result here.

We also have now analyzed the efficiency of high- and low-LET paternal exposure on the transgenerational instability in the F₁ and F₂ offspring within CBA/H inbred mice. High-LET radiation produces highly complex and localized initial DNA damage, which is qualitatively different to the sparse damage produced by low-LET radiation. It has been proposed that these differences in damage patterns may result in the unique final biological effects of these different radiation sources (21). However, it seems that both irradiations are capable of inducing genomic instability in somatic cells, although some studies have failed to detect the effects of low-LET exposure (reviewed in ref. 22). Our results show that paternal exposure to either high-LET fission neutrons or low-LET x-rays results in increases in germ-line mutation rates in both generations and therefore are consistent with the data on somatic cells.

In a number of our previous studies we have shown that postmeiotic exposure to ionizing radiation does not affect ESTR germ-line mutation rate in the directly exposed males (5, 7). The lack of mutation induction at this stage has been explained by assuming that spontaneous and radiation-induced mutation at ESTR loci most probably occurs in diploid cells but not in haploid postmeiotic cells. In contrast, we now have shown that a similar elevated mutation rate is found in the germ line of F₁ offspring of exposed males conceived from either postmeiotic (3 weeks) or premeiotic (6 weeks) stages of spermatogenesis, and this elevated mutation rate persists to the germ line of the second generation (Fig. 3*b*). Thus, the destabilization of the F₁ germ line occurs after fertilization regardless of the stage of spermatogenesis exposed to radiation, and the radiation-induced signal also persists and destabilizes the F₂ germ line. Several recent publications report conflicting data on the efficiency of postmeiotic paternal exposure and the effects that it has on the transgenerational mutation processes (23–28). This apparent discrepancy in findings remains unexplained, and future work should directly address in greater detail the issue of the stage specificity of transgenerational effects.

The data presented here show elevated germ-line mutation rates in the F₁ and F₂ offspring of initially irradiated males belonging to three inbred strains CBA/H, C57BL/6 and BALB/c, which clearly demonstrates that transgenerational instability is not restricted to one particular inbred strain of mice and shows significant interstrain variation in transgenerational instability. Previous studies of these three inbred strains of mice also have revealed profound differences in their response to ionizing radiation, showing that BALB/c and CBA mice are significantly more radiosensitive and show higher levels of radiation-induced genomic instability in somatic cells than C57BL/6 mice (12, 14–17). It has been suggested also that the high level of radiation-induced genomic instability in BALB/c mice could be attributed to the strain-specific amino acid substitutions at the *Cdkn2a* and *Prkdc* genes (11, 12). Our data

Table 2. Transgenerational changes in the offspring of male mice and rats exposed to ionizing radiation or chemical mutagens

Strain	Paternal exposure	Generation	Endpoint	Effect*	Ref.
Mice					
ICR	X-rays (216 rad)	F ₁	Lung tumors after treatment with urethane	Elevated	29
SHR	X-rays (4.2 Gy)	F ₁ , F ₂	Skin tumors after treatment with phorbol 12-myristate 13-acetate	Elevated	27
CBA/H	Injection with ²³⁹ Pu	F ₁	Lympho-hemopoietic malignancies after treatment with methylnitrosourea	Elevated	30
DBA2	Injection with ⁵⁵ Fe	F ₁	Lympho-hemopoietic malignancies after treatment with methylnitrosourea	Elevated	23
CBA	Injection with ²³⁹ Pu	F ₁	Pre-/postimplantation loss among offspring	Elevated	18
HLG/Zte	X-rays (1 Gy)	F ₁	Pre-/postimplantation loss and malformations among offspring	Elevated	19
CBA/H	Neutrons (0.4 Gy)	F ₁	Germ line mutation rate at two ESTR loci	Elevated	3
C57BL/6	X-rays (1–4 Gy)	F ₁	Somatic mutation rate at <i>lacI</i> gene	Elevated	26
C57BL/6J	X-rays (1 Gy)	F ₁	Somatic reversions at <i>p^u</i> locus	Elevated	31
CD1	γ-rays (1 Gy)	F ₁	Proliferation of early embryonic cells in offspring	Decreased	24
CD1	γ-rays (1 Gy)	F ₁	Fertilization rate for spermatozoa	Decreased	25
CD1	γ-rays (1 Gy)	F ₃	Levels of p53 and p21 ^{waf1}	Elevated	32
Rats					
Sprague	Cyclophosphamide	F ₁	Pre-/postimplantation loss and malformations among offspring	Elevated	20
Swiss	X-rays (4.5 Gy)	F ₁	Chromosome aberrations after treatment with x-rays or cyclophosphamide	Elevated	28
Wistar	Cyclophosphamide	F ₁ , F ₂	Learning capacity	Decreased	33

*Compared with the offspring of nonexposed parents.

showing low spontaneous germ-line mutation rate and radiation-induced transgenerational instability within the C57BL/6 strain with significantly higher values for the CBA/H and BALB/c strains are consistent with these observations.

The results of this study also provide important clues relating to the possible mechanisms of transgenerational instability. If the transgenerational signal inducing instability in the F₁ germ line was direct radiation-induced DNA damage within the ESTR loci themselves, then mutations in the F₁ germ line should occur predominantly in the damaged allele transmitted to them from the irradiated F₀ fathers. Because equally elevated mutation rates at both alleles derived from the irradiated F₀ fathers and the unexposed F₀ mothers were detected in the germ line of F₁ offspring, this explanation can be excluded, and a global elevation of mutation rate is inferred. The persistence of elevated mutation rates in the germ line of two consecutive generations rules out the possibility that transgenerational effects are caused by radiation-induced mutations at any specific set of genes in the exposed F₀ males. For example, mutations in some DNA-repair genes transmitted from irradiated males to their offspring potentially could affect genome stability in the first generation. However, the further mating of F₁ offspring to control animals should result in Mendelian segregation of wild-type and mutant alleles, causing an overall reduction of mutation rate in the

second generation. On the contrary, mutation rates in the germ line of both generations are similar and significantly exceed those for the control parents, clearly implicating an epigenetic mechanism for the transgenerational instability.

Finally our results may have far-reaching implications for the evaluation of genetic risks of ionizing radiation. If germ-line instability is able to persist in populations for several generations after the initial exposure to ionizing radiation, this instability would lead to a significant increase in mutation load. Because ESTRs are noncoding loci with no apparent function, the germ-line mutations studied here can be considered selectively neutral, and it may be possible to argue that instability at these loci should not affect the fitness of population. However, recent studies provide strong evidence for health-related transgenerational effects, affecting predisposition to cancer, mortality, somatic mutation, fertility, and behavior (Table 2). Altogether, these data raise the important issue of the delayed transgenerational effects of ionizing radiation for humans, providing, for example, a plausible explanation for the apparent leukemia cluster near Sellafield nuclear plant (34).

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