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REVIEW

Assessing Radiation-Associated Mutational Risk to the Germline: Repetitive DNA Sequences as Mutational Targets and Biomarkers

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This review assesses recent data on mutational risk to the germline after radiation exposure obtained by molecular analysis of tandemly repeated DNA loci (TRDLs): minisatellites in humans and expanded simple tandem repeats in mice. Some studies, particularly those including exposure to internal emitters, indicate that TRDL mutation can be used as a marker of human radiation exposure; most human studies, however, are negative. Although mouse studies have suggested that TRDL mutation analysis may be more widely applicable in biomonitoring, there are important differences between the structure of mouse and human TRDLs. Mutational mechanisms probably differ between the two species, and so care should be taken in predicting effects in humans from mouse data. In mice and humans, TRDL mutations are largely untargeted with only limited evidence of dose dependence. Transgenerational mutation has been observed in mice but not in humans, but the mechanisms driving such mutation transmission are unknown. Some minisatellite variants are associated with human diseases and may affect gene transcription, but causal relationships have not yet been established. It is concluded that at present the TRDL mutation data do not warrant a dramatic revision of germline or cancer risk estimates for radiation. **© 2006 by Radiation Research Society**

INTRODUCTION

There is a lack of positive evidence indicating an increase in human germline mutation after exposure to radiation from the atomic bombings in Japan (*1*). Consequently, current genetic risk estimates for radiation are based on measurement of mutation frequencies in the mouse using a handful of ''marker genes'' (*2, 3*). For example, mutations in genes controlling mouse fur color can be recognized easily and show a dose-dependent increase after irradiation. However, the naturally occurring frequency of such gene mutations is about 1 in 100,000 mice, necessitating very large-scale experiments to determine frequency increases. Based on this relatively low frequency of mutations and extensive data from somatic mammalian cells, it was considered that ionizing radiation-induced mutations arose at or close to the sites of damage.

In recent years, analysis of mouse and human DNA sequence has shown that some regions containing repetitive sequence elements, collectively known as tandemly repeated DNA loci (TRDLs), mutate at a much higher frequency ($\times$1000) than the well-known marker genes (*4*). Although the functions, if any, of these highly mutable sequences are not well understood and may differ between mice and humans, they may nevertheless act as markers of mutation induction. Additionally, the very high frequencies of radiation-induced mutation observed in mice suggest that these mutations are not linked directly to sites of DNA damage; i.e., the mutations are ''untargeted''. Other types of experiment have shown that radiation may also cause changes in cells, including mutations, long after the radiation exposure has ended [see, for example, ref. (*5*)]. The mechanisms of these non-targeted and delayed effects of radiation are not well understood, although it is assumed that some radiation-induced event triggers these sequences to become genetically less stable.

Analysis of TRDLs provides an important new method for following mutations in the mouse and human germlines (*6*). In humans, the highly mutable sequences occur at cer-

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TABLE 1
Average Spontaneous Mutation Rates at the Most Unstable Human Minisatellite Loci Used to Study Mutation[a]

Probe	Locus	Repeat size (bp)	Mutation rate per locus (range)		Ratio paternal to maternal
			Paternal	Maternal	
B6.7	(20q13)	34	0.063 (0.036–0.088)	0.020 (0–0.038)	3.2
CEB1	*D2S90*	39	0.127 (0.101–0.144)	0.004 (0–0.012)	36.5
CEB15	*D1S172*	18	0.031 (0.014–0.056)	0.004 (0–0.012)	8.7
CEB25	*D10S180*	52	0.038 (0.027–0.065)	0.007 (0–0.012)	5.3
CEB36	*D10S473*	42	0.008 (0–0.015)	0.014 (0–0.025)	0.5
MS1	*D1S7*	9	0.042 (0.021–0.090)	0.049 (0.034–0.080)	0.8
MS31	*D7S21*	20	0.012	0	—
MS32	*D1S8*	29	0.010	0	—
Mean	—	—	0.043	0.012	3.8

[a] Data from Dubrova *et al.* (*17–19*); Livshits *et al.* (*20*), Kiuru *et al.* (*21*), and Kodaira *et al.* (*22*).

tain ''minisatellites'', whereas in the mouse germline, high mutation frequencies have been found in certain expanded simple tandem repeats (ESTRs). Although ESTRs were formerly termed minisatellite sequences, there are important structural differences between mouse ESTRs and human minisatellites as described below.

This review summarizes the information currently available from molecular genetic and related studies of germline mutation largely in males. It reflects the outcome of a review undertaken by the authors for the UK Committee on Medical Aspects of Radiation in the Environment (COMARE). The opinions expressed are, however, those of the authors and are not necessarily those of COMARE. The review considers mechanisms of mutation and possible health effects of TLDR mutation as well as radiation exposure monitoring studies. Critical issues are:

1. the scope, strength and significance of the human and mouse studies of radiation-induced TRDL mutation;
2. the relationship between the mouse and the human data;
3. the potential impact of these mutations on human health.

BIOLOGY OF TANDEMLY REPEATED DNA LOCI

Tandemly repeated DNA loci in the mammalian genome are represented by relatively short microsatellites (<500 bp) with repeat sizes of 1–4 bp, long expanded simple tandem repeats (ESTRs, 0.5–16 kb, repeat sizes 4–9 bp), and true minisatellites (0.5–10 kb) with repeat sizes of 9–60 bp [reviewed in refs. (*4, 7, 8*)]. Tandem repeat DNA may constitute as much as 10% of the human genome. In contrast to mutations in functional genes, the majority of mutations at these repeat loci are represented by gains and losses of entire repeat units. Analysis of mutation at minisatellite and ESTR loci has been used to examine the effects of radiation on the germline.

Minisatellite Loci

Minisatellites consist of 9–60-bp repeats that show considerable sequence variation along the array (*8–12*). Minisatellites have been detected in the genomes of most higher eukaryotes, including mice and humans. Human minisatellites are found predominantly in sub-telomeric regions. However, in the pig, rat and mouse genomes, they show less telomeric clustering (*13, 14*). In addition, germline mutation rates at human minisatellite loci are substantially higher than those in the pig, rat and mouse genomes (*15*).

To date, minisatellite mutation rates in humans have been evaluated using either the pedigree approach (*16*) or the small-pool PCR technique (*10–12*). The pedigree approach is based on mutation scoring in full families (mother-father-child/children). DNA samples are most often extracted from peripheral blood lymphocytes, and minisatellite loci are then detected by hybridization with locus-specific probes. Mutations are identified as novel DNA fragments present in the offspring that cannot be ascribed to either parent. Given the high mutability of these loci, the parental alleles are nearly always different so that the parental origin of mutations is practically always possible to establish. The pedigree analysis of each family should always include checks to exclude non-paternity or sample mix-up. The small-pool PCR approach is based on the amplification of multiple diluted aliquots of sperm DNA and allows the detection of large numbers of *de novo* mutations in a single male (*10*). It has been used mainly for the analysis of mechanisms of minisatellite mutation in the paternal germline (*10–12*). The pedigree-based analysis of minisatellite loci remains the only source of experimental data on minisatellite mutation in the maternal germline. In this review, mutation rate is defined as the number of mutations passed on to the next generation by an individual in a sample of mice, humans or another population. It is calculated by dividing the total number of mutations scored in the offspring by the total number of parental TRDL bands and corresponds to the mutation frequency per generation.

Mutation rates at minisatellites are locus-specific and show considerable variation between the paternal and maternal germlines (Table 1). For the majority of human minisatellite loci, an excess of paternal mutations has been

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TABLE 2
Spontaneous Mutation Rates at the Most Unstable Mouse ESTR Loci[a]

	Ms6-hm $(GGCAG)_n$			*Hm-2* $(GGCA)_n$			*Ms6-hm* + *Hm-2*		
Strain	Paternal	Maternal	Ratio[b]	Paternal	Maternal	Ratio[b]	Paternal	Maternal	Ratio[b]
CBA/H	0.087	0.087	1.00	0.040	0.047	0.86	0.063	0.067	0.95
CBA/Ca	0.041	0.074	0.56	0.058	0.066	0.88	0.050	0.070	0.71
C57BL/6	0.122	0.061	2.00	0.010	0.061	0.17	0.066	0.061	1.08
F_1[c]	0.100	0.079	1.27	0.063	0.068	0.92	0.082	0.074	1.11
BALB/c	0.181	0.085	2.13	0.074	0.138	0.54	0.128	0.112	1.14
Mean	0.101	0.078	1.29	0.050	0.072	0.70	0.076	0.075	1.01

[a] Data from Dubrova *et al.* (*40*), Barber *et al.* (*41, 42*), and Vilarino-Guell *et al.* (*43*).
[b] Ratio of paternal mutation rate to maternal.
[c] C57BL/6 × CBA F_1.

found. On average, the minisatellite mutation rate in the paternal germline is about four times higher than in the maternal germline. A similar sex bias has also been reported for human microsatellite loci (*6*). The reason for such a bias at minisatellite loci remains unknown. It should be noted that for some minisatellite loci, the opposite trend has been reported (Table 1).

Analyses of minisatellite loci have shown that the mutational processes in somatic tissue and the germline are qualitatively and quantitatively different. High-frequency mutation at these loci is almost completely confined to the germline, with very rare and simple mutational events occurring in the somatic cells (*10, 12, 23, 24*). Germline mutation at human minisatellites may be attributed to complex gene conversion-like events, often resulting in multiple mutational changes. No single model has yet been developed to account for all the different types of minisatellite mutation in the germline. It has been suggested that minisatellite mutations may be initiated by DNA double-strand breaks. Alternatively they may arise as a consequence of staggered nicks that extend into the repeat array. This could be followed by single-strand DNA invasion by the broken DNA strand into the other minisatellite allele or into a sister chromatid, leading to intra-allelic exchange (*10*). It has also been shown that the mutation process at some minisatellite loci may be driven by an adjacent hotspot of meiotic recombination (*25–27*).

Some minisatellites, possibly up to 30–40% of the total (*28*), have been identified in coding regions of the human genome where variations in the number of minisatellite repeats could affect their structure. Examples include members of the apolipoprotein gene family (*29*) and human epithelial mucin (*30*). The presence of minisatellites in the vicinity of the *HRAS* gene could affect its transcription and may predispose to heritable forms of cancer (*31*). Minisatellites within intronic regions of genes have been shown to interfere with exon splicing; this may be due to the similarities between the consensus repeat of the minisatellite and splice donor sites, as is seen for the human interferon-inducible gene 6–16, *G1P3* (*32*). Although the role of minisatellite loci remains unclear, the large number of loci, their persistence in the genome, and the fact that they are found within the genomes of most eukaryotic organisms would suggest that they possess some function.

Expanded Simple Tandem Repeat (ESTR) Loci

ESTR loci were originally termed minisatellites but have recently been renamed to distinguish them from the much more stable true minisatellites in the mouse genome (*33, 34*). Unstable ESTRs consist of homogeneous arrays of relatively short repeats (4–9 bp), and, in contrast to minisatellites, ESTRs show a very high spontaneous mutation rate in both germline and somatic cells (*34–36*). Currently ESTRs have been identified only in the mouse, but they are thought to be present in other genomes, including that of humans. It has been shown that ESTR loci are derived from highly expanded interspersed repeat elements in the mouse (*34*). In contrast to human minisatellites, mouse ESTR loci do not show any preferential telomeric clustering and are distributed more or less randomly in the mouse genome (*34*).

To date, ESTR mutation rates in the mouse germline have been evaluated using the pedigree approach [reviewed in refs. (*37, 38*)]. However, the results published in a recent report show that a novel single-molecule PCR approach can provide robust estimates of the frequency of ESTR mutation in mouse sperm and somatic tissues (*39*).

In contrast to human microsatellites and minisatellites, spontaneous ESTR germline mutation rates in mice do not exhibit an extensive paternal bias (Table 2). The high somatic mutation rate at ESTR loci leads to high levels of mosaicism; e.g., between 2.8% and 20% of adult mice possess more than two alleles at the mouse ESTR loci *Ms6-hm* and *Hm-2* (*35, 36*). Further analyses of somatic tissues during different stages of development have demonstrated that the high levels of somatic mutation at the *Ms6-hm* and *Hm-2* loci take place during the early stages of embryogenesis (*35, 36*).

It has been proposed that spontaneous ESTR mutation is most probably attributable to replication slippage, with similar mechanisms being suggested for microsatellite instability (*38, 39, 44*). According to this model, the very high spontaneous mutation rates at some ESTR loci could be

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TABLE 3
Properties of Tandem Repeat DNA Loci

	Minisatellites	ESTRs[a]	Microsatellites
Repeat unit	10–60 bp	4–10 bp	2–6 bp
Size of array	500 bp–15 kb	100 bp–20 kb	10 bp–1 kb
Complexity of array	Heterogeneous	Mostly homogeneous	Mostly homogeneous
Genomic distribution			
Mouse	Random?	Random?	Random
Humans	Sub-telomeric	?[b]	Random
Somatic instability	Very low	High	High
Mutation mechanism			
Germline	Meiotic recombination	Replication slippage?	Replication slippage
Somatic cells	?[b]	Replication slippage?	Replication slippage

[a] Expanded simple tandem repeats.
[b] Unknown.

directly related to their very large size (500–3500 repeats) and probably to the presence of hairpin structures within the arrays (*45, 46*). These together may cause replication pausing and could subsequently promote DNA polymerase slippage. This model is consistent with the observed positive correlation between the spontaneous germline mutation rates and the sizes of ESTR loci (*47*).

To summarize, Table 3 presents a comparison of the three types of tandem repeat DNA loci detected to date in humans and mice. It appears that the mutational behavior of minisatellite loci differs dramatically from that of ESTRs and microsatellites. It therefore follows that the use of mouse ESTR loci as models for human minisatellite instability should be treated with considerable caution.

STUDIES OF MINISATELLITE MUTATION IN HUMANS

As described above, minisatellites constitute the most unstable loci in the human genome, with mutation rates ranging from 0.5 up to 13% per gamete per generation. Mutation at these loci is almost completely restricted to the germline and most probably occurs during meiosis. Given the meiotic origin of spontaneous minisatellite mutation, it appears likely that mutation induction by radiation at these loci is attributable to radiation-induced changes in premeiotic diploid germ cells that subsequently affect the stability of minisatellite loci at meiosis. If so, then intrinsic differences in the timing of spermatogenesis and oogenesis could exert a profound influence on the patterns of minisatellite mutation induction in the paternal and maternal germlines. Spermatogenesis is a continuous process of mitotic and meiotic cell divisions, and there is some evidence to suggest that minisatellite mutation rates may be elevated after the irradiation of diploid spermatogonia of adult men. By contrast, oocytes are already formed in late embryogenesis and remain arrested until the onset of puberty, so that minisatellite mutation induction in the maternal germline may only be detected in a cohort of females irradiated during the early stages of gestation. Overall, the human data are at present weak, and no firm conclusions may be drawn on the frequency of minisatellite mutation after radiation exposure.

To date, using the pedigree approach, germline minisatellite mutation rates have been evaluated in four irradiated groups from Japan and the former USSR.

The Hiroshima and Nagasaki Atomic Bomb Survivors

Minisatellite mutation rates have been evaluated in exposed and control families from Hiroshima and Nagasaki (*22, 48*). Key features of these studies:

1. A relatively small group of 62 children (30 of exposed fathers and 32 of exposed mothers) and 60 children of non-exposed parents were analyzed.
2. The doses of parental exposure were reconstructed using Dosimetry System 86 (DS86).
3. The exposed group with mean parental dose of 1.9 Sv mainly comprised families with only one irradiated parent.
4. Most of the children from the exposed families were born $>$10 years after the bombings.
5. Four of the six probes used in the first study detected loci with a very low mutation rate (*48*).
6. In the second study, germline mutations were scored using eight single-locus probes detecting the most unstable human minisatellite loci (*22*).

These studies failed to detect any significant changes in mutation rates in the germlines of exposed families. This failure may be attributable to (1) the small sample sizes, (2) a high proportion of families containing irradiated mothers and nonirradiated fathers, (3) the successful repair of radiation-induced damage over a long period prior to conception, and (4) a truly low induced mutation frequency.

Populations in the Heavily Polluted Rural Areas around the Chernobyl Nuclear Plant

Three publications have presented analyses of minisatellite mutation rates in post-Chernobyl families from rural

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areas of Ukraine and Belarus (*17, 19, 49*). In the Belarus study (*17, 49*):

1. 127 children of irradiated parents from the Mogilev region of Belarus and 120 children of nonexposed Caucasian parents from the UK were analyzed.
2. Control and exposed groups were not matched.
3. Germline mutations were scored using two multilocus and eight single-locus probes that detect the most unstable human minisatellite loci.

The results of this study were interpreted as providing evidence that mutation rates in the Belarus cohort were elevated for most of the minisatellite loci analyzed. Moreover, within the exposed cohort, the mutation rate was found to be significantly greater in families with higher parental radiation dose estimated for chronic external and internal exposure to cesium-137 (mean 27.6 ± 3.3 mSv; median = 19.7 mSv, $P = 0.015$), consistent with the radiation induction of germline mutation. It should be stressed, however, that the cesium-137 dose reflects only one, and perhaps a minor, component of radiation exposures received by those inhabiting Chernobyl-contaminated land. Given that mutation rates in the exposed group were compared with those in nonexposed Caucasian families of different ethno-geographic origin, the results of this study do not provide sufficient evidence to support the contention that the germline mutations were induced by radiation.

To verify the results of this study, minisatellite mutation rates were evaluated in exposed and nonexposed families from rural areas of the Kiev and Zhitomir regions of Ukraine (*19*). In this study:

1. 240 children of the irradiated parents and 98 children of nonexposed parents born in the same area before the Chernobyl accident were analyzed.
2. Control and exposed groups were matched by ethnicity, maternal age, parental occupation and smoking habit and differed only slightly by paternal age.
3. Germline mutations were scored employing the same eight single-locus probes used in the Belarus study.

The results of this study are indicative of a statistically significant elevated paternal mutation rate ($P = 0.03$) in the exposed families from Ukraine and Belarus but do not provide evidence for elevated mutation rates in the germlines of exposed mothers. The similarity in mutation rates in the germlines of exposed and nonexposed mothers could be attributed to the fact that none of the mothers included in the Ukrainian and Belarus studies had been irradiated *in utero* during the meiotic stages when minisatellite mutation can occur. Taken together, the two studies provide evidence that the germline minisatellite mutation rate in families inhabiting rural areas of Belarus and Ukraine that were heavily contaminated by radionuclides after the Chernobyl accident is indeed elevated. The authors also suggested that the elevated paternal mutation rate found in the Ukrainian and Belarus cohorts of exposed families may be attributed to the high initial exposure during the first days after the Chernobyl accident. Using published data on the reconstruction of the likely doses of exposure for the residents of heavily contaminated areas of Belarus and Ukraine (0.2–0.4 Gy), the authors concluded that the 1.6-fold increase in minisatellite mutation rate found in the families from Ukraine and Belarus is broadly consistent with estimates of the doubling dose for germline mutation in humans, i.e. 1 Gy (*50, 51*).

The Families of Chernobyl Cleanup Workers

In three recent studies, the frequency of minisatellite mutation was analyzed in the offspring of Chernobyl cleanup workers from the Ukraine (*20, 52*) and Estonia (*21*). In all studies, the effects of paternal exposure to ionizing radiation were analyzed. In the study of Livshits *et al.* (*20*):

1. 183 children of irradiated parents and 163 children of nonexposed parents from southern Ukraine were analyzed.
2. No description of the control group was given. It is therefore unclear whether the control and exposed groups were matched or not.
3. Germline mutations were scored employing seven single-locus probes that detect the most unstable human minisatellite loci.

In the study of Slebos *et al.* (*52*):

1. 51 children from 51 families born after paternal radiation exposure were examined.
2. The controls were 24 children born to a subset (24) of the above families before paternal radiation exposure.
3. Germline mutations were scored using multilocus minisatellite probes 33.6 and 33.15 and six single-copy microsatellite markers.

In the Estonian study (*21*):

1. 148 children of irradiated parents and 155 children of nonexposed parents were analyzed.
2. The control group consisted of children conceived by the same parents before the Chernobyl accident.
3. Germline mutations were scored using the same eight single-locus probes used by Dubrova *et al.* (*17–19*).

The results of these three studies were almost identical and failed to reveal any increases in the minisatellite mutation rate in the germline of irradiated fathers. It should be stressed that the doses for this group of workers are thought to be extremely heterogeneous, although most received doses of less than 0.25 Gy, with a dose range of 0.01–1 Gy (*53*). Importantly, the group of Chernobyl cleanup workers was exposed to repeated small daily doses of ionizing radiation. The relatively low-dose exposure of these Chernobyl cleanup workers [mean dose 0.11 Sv; see ref. (*21*)], suggests that the expected increase in mutation rate in this group may be too small to detect. Although the

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study of Slebos *et al.* (*52*) did detect a modest increase in microsatellite mutation frequency in children of exposed fathers, the increase did not reach statistical significance. The results of a more recent study failed to provide any evidence for a measurable increase in the microsatellite mutation rate in the germlines of Chernobyl cleanup workers (*54*).

A further study reported a sevenfold increase in mutation rate in a cohort of 42 children of ''liquidators'' (cleanup workers) involved in the immediate response to the Chernobyl incident (*55*). In this study, Weinberg *et al.* used short random-sequence PCR primers to amplify DNA segments from the human genome [an unvalidated technique for monitoring germline mutation in humans; see ref. (*56*)]. Given that the doubling dose for mammalian germline mutation has been estimated as 1 Gy (*50*), such an increase would imply that the cohort studied has been exposed to doses of up to 6 Gy, a whole-body dose that is generally lethal. In fact, most of those participating in the decontamination work around the Chernobyl nuclear power plant, sarcophagus construction and other cleanup operations received doses of external exposure of less than 0.25 Gy (*53*). Clearly, these findings contrast with the negative results of three large studies on TRDL mutation rates in Chernobyl cleanup workers (*20, 21, 52, 54*).

Nuclear Weapon Tests and Minisatellite Mutation Rates

Minisatellite mutation rates were also evaluated in the germlines of irradiated parents living around the Semipalatinsk nuclear test site (*18*). Semipalatinsk was the site for 470 nuclear tests performed by the Soviet Union during the period 1949–1989, including atmospheric and surface explosions (1949–1963) and underground tests (1963–1989). The surrounding population was exposed mainly to the fresh radioactive fallout from four surface explosions conducted between 1949 and 1956; currently, the radioactive contamination outside the test zone is low. In this study:

1. The exposed group comprised 40 three-generation families inhabiting the rural areas of the Semipalatinsk district of Kazakhstan around the Semipalatinsk nuclear test site.
2. The control group was composed of 28 three-generation nonirradiated families from the geographically similar rural area of the former Taldy Kurgan district of Kazakhstan, which was not contaminated by nuclear tests.
3. Control and exposed groups were matched by ethnicity, maternal age, parental occupation and smoking habit.
4. Germline mutations were scored by employing the same eight single-locus probes used by Dubrova *et al.* (*17, 19*).

The results of this study indicate that exposure to radioactive fallout from the nuclear weapons tests carried out at the Semipalatinsk nuclear test site in the late 1940s to early 1950s roughly doubled the germline mutation rate in the exposed population. Importantly, in the cohorts of parents exposed to lower doses of ionizing radiation after the decay of radioisotopes in the late 1950s after the cessation of surface and atmospheric nuclear tests, a negative correlation between the mutation rate and parental year of birth was found. Therefore, despite the lack of reliable data on the doses received by this cohort, this correlation could imply the presence of a dose–response relationship for minisatellite mutation induction and suggests that an elevated mutation rate in the affected families is indeed induced by radiation.

Cancer Chemotherapy and Radiotherapy Patients

A novel small-pool PCR (SP-PCR) approach for the detection of minisatellite mutations has been used to analyze mutation induction in the germlines of male cancer patients (*57–59*). This approach is based on the amplification of multiple diluted aliquots of sperm DNA and allows the detection of a large number of *de novo* mutations in a single male (*10*). Compared to the pedigree approach, this technique dramatically reduces the number of individuals needed for the measurement of germline mutation frequencies. The major shortcoming of the SP-PCR approach is a very high degree of variation between spontaneous mutation rates of individual alleles at a single locus (*10–12*), effectively precluding comparisons of mutation rates between groups of exposed and nonexposed men. Therefore, this technique can only be used to evaluate the mutation rate in the same man before and after therapeutic treatment with agents that damage DNA. Moreover, SP-PCR does not allow amplification of very large minisatellite alleles (longer than 5 kb), thus restricting mutation scoring to a subset of relatively small alleles.

Using SP-PCR of the MS205 minisatellite, germline mutation rates were determined in cancer patients treated with therapeutic mutagens (*57, 59*). In the first study, sperm samples from two men, collected before and after treatment with the anticancer drugs cyclophosphamide, etoposide and vincristine, were analyzed, and no effects on the minisatellite mutation rate were detected (*57*). The alkylating agent cyclophosphamide induces a variety of germ cell effects in post-meiotic stages only, with no effects on pre-meiotic stages (*60*). The topoisomerase II inhibitor etoposide affects only meiotic germ cells and is not mutagenic in pre- and post-meiotic cells (*43, 62*). If correct, then the time window for mutation induction by this drug is very short and may be difficult to analyze. Vincristine prevents the assembly of tubulin into spindle fibers, and there is no indication of germ cell mutagenicity for this drug in mice (*60*). Therefore, the two men analyzed in the above-mentioned study (*57*) were exposed to the anticancer drugs that, because of the stage specificity of mutation induction in the male germline, may not induce mutation at minisatellite loci at all or, alternatively, could affect a very small subset of germ cells over a relatively short period.

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In the second study, sperm samples collected from 10 men before and after treatment for Hodgkin's disease were analyzed (*59*). Nine patients who were treated either with vinblastine or with Adriamycin and bleomycin did not show any increases in mutation rate after cancer chemotherapy. Vinblastine binds to tubulin, and exposure of male mice to this drug results in aneuploidy rather than chromosome breakage or gene mutation (*60*). Adriamycin is an intercalating agent and an inhibitor of topoisomerase II. Exposure to Adriamycin results predominantly in cell toxicity in mouse germ cells, without mutation induction (*60*). Bleomycin, a radiomimetic drug, selectively targets mouse oocytes; mutation induction has not been observed in male germ cells (*60*). Therefore, judging from the mouse data, the negative results for the cancer patients are not unexpected. Interestingly, the only patient treated with procarbazine, a powerful pre-meiotic mutagen in mice (*60*), exhibited a significant increase in mutation rate after chemotherapy (*59*).

The analysis of sperm DNA from three seminoma patients before and after radiotherapy also failed to detect any increases in mutation rate at the hypervariable minisatellites B6.7 and CEB1 (*58*). These men were repeatedly exposed to 15 fractions of acute X rays with a total testicular dose ranging between 0.4 and 0.8 Gy, a value close to the estimates of doubling dose in male mice (*50, 51*). It should be noted that the negative results of the study of May *et al.* (*58*) may be attributable to several factors, including the possibility that dose fractionation reduces mutation rates at repeat loci as it does at single-copy gene loci (*61*).

Conclusions from Human Studies

The analysis of minisatellite loci can potentially provide a system for monitoring germline mutation in humans. The main advantage of this system is its ability to detect changes in mutation rate in relatively small population samples, a consequence of the very high spontaneous mutation rates at these loci. Using this system, the first experimental evidence was obtained that the germline mutation rate in a human population may be increased after ionizing radiation exposure. However, since the human experimental data have so far been derived from relatively small numbers of families, additional surveys are needed to evaluate the extent of radiation-induced minisatellite mutation in our species. Importantly, not all exposed populations have been found to display elevated mutation rates. Specifically, TRDL mutation rates were increased in humans from areas highly contaminated with internal emitters but not in populations exposed predominantly to external radiation sources. This may be due in part to the lack of reliable radiation dosimetry, which limits the application of minisatellites as a reliable system for monitoring radiation-induced mutation in humans. It should also be noted that human population studies are subject to the same confounders as any other environmental monitoring studies, for example, exposure to chemical contaminants in addition to radiation (see the section on environmental monitoring studies below). Studies showing an elevated mutation rate in irradiated families from Belarus, Ukraine and Kazakhstan (*17–19, 49*) have failed to establish any reliable relationship between radiation dose and mutation rate. Given that germline mutagenicity studies require an evaluation of the dose–response parameters, future studies are essential to address this important issue.

RADIATION INDUCTION OF ESTR MUTATION IN GERM CELLS OF MALE MICE

Radiation-induced mutation in highly unstable expanded simple tandem repeat (ESTR) DNA sequences in male germ cells of mice was first reported in the early 1990s (*63, 64*). ESTR sequences consist of long arrays of 4, 5 or 6 DNA base-pair repeats that can show spontaneous germline mutation frequencies as high as 10% per gamete per generation (*65*). The mutations are seen as size changes in the array; both increases and decreases in the number of repeat units occur, with a slight bias toward increases in size (*38, 65*). Most studies have used the same specific sequences (the 5-bp repeat *Ms6-hm* on mouse chromosome 4 and the 4-bp repeat *Hm-2* mapping to mouse chromosome 9), although early studies made use of ''multilocus'' probes that detected several different repeat sequences including *Ms6-hm* and *Hm-2*. Since the doses of radiation used are unlikely to damage the ESTR sequences directly (owing to their relatively small size, representing about 1 part in 200,000 of the mammalian genome), it was concluded that mutations arise from some form of radiation-induced genetic instability leading to non-targeted mutational events (*64, 67, 68*).

Radiation-induced ESTR mutation in the mouse has been examined under different conditions, such as at different doses and dose rates, with different radiation qualities (sparsely and densely ionizing radiation), and at different stages of mouse spermatogenesis. In all studies, the ESTR mutation rate in the germline was estimated from the frequency of ESTR mutation in the offspring of control and irradiated mice. Table 4 summarizes the published studies and their conditions.

Dose Responses, Dose-Rate Effects and Doubling Doses

There is some evidence for a dose response over a range of doses from 0.5 to 2 Gy acute X rays or γ rays (*38, 67, 71*). While average mutation rates increased by three- to fourfold at the highest radiation dose used, it is of some concern that the variation in mutation rate found between individual mouse families was equally large (three- to fourfold) at a given dose. Similar data were reported for more densely ionizing neutron irradiations; where sufficient data have been reported, the relative effectiveness of fission neu-

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TABLE 4
Summary of Published Studies on Germline ESTR Mutation in Mouse, Modified from Niwa (*69*)

Male mice	Female mice	Radiation type; dose (dose rate)	Mating weeks postirradiation	Locus/probe	Main observations
101/HY × C3H/SnY[a]	101/HY × C3H/SnY	γ rays; 0.5, 1 Gy	6	Multilocus/33.6, 33.15	1.5–1.9× increased mutation rate doubling dose 0.5 Gy
C3H/HeN[b]	C57BL6N	γ rays; 3 Gy (0.5 Gy/min)	0–1, 2–3, 10–11	Single locus/Mh6-hm	Spermatids sensitive
C3H/HeN[c]	C57BL/6N	γ rays; 1, 2, Gy (0.5 Gy/min)	0–1, 2–3, 10–11	Single locus/Mh6-hm	Doubling dose of 0.83 Gy for spermatids
C3H/HeN[d]	C57BL/6N	Neutrons; 0.35, 0.7, 1 Gy (0.006 Gy/min)	0, 2, 10	Single locus/Mh6-hm	RBE of 2.6 spermatid
CBA/H[e]	CBA/H	X rays; 0.5, 1 Gy (0.5 Gy/min)	3, 6, 10	Multilocus/MMS10, 33.15; single locus/Mh6-hm, Hm2	Postmeiotic stage insensitive. Doubling dose of 0.33 Gy for stem cells and spermatogonia
C57BL/6 × CBA/H[f]	CBA/H	X rays; 1 Gy (0.5 Gy/min)	3, 4, 5, 6	Multilocus/MMS10; single locus/Mh6-hm, Hm2	Postmeiotic stage insensitive
CBA/H[g]	CBA/H	X rays; 0.5, 1 Gy (0.166 mGy/min). Neutron; 0.125, 0.25, 0.5 Gy (3 mGy/min)	10	Multilocus/MMS10; single locus/Mh6-hm, Hm2	No dose-rate effect for X rays. RBE of 3.4 for neutrons
C57BL/6N[h]	C3H/HeN	γ rays; 6 Gy (0.5 Gy/min)	0–1	Single locus/Mh6-hm	Maternal allele mutated by spermatozoa irradiation
CBA/H, C57BL and BALB/c[i]	CBA/H, C57BL & BALB/c	X rays; 1 Gy (0.5 Gy/min). Neutron; 0.4 Gy (3 mGy/min)	3, 6	Multilocus/MMS10; single locus/Mh6-hm, Hm2	Transgenerational instability
CBA/H, C57BL/6, BALB/c and C.B17[j]	CBA/H, C57BL/6, 129SVJ and BALB/c	X rays; 0.5, 1 Gy (0.5–2 Gy/min)	4–10	Single locus/Mh6-hm, Hm2	Similar mutation rates in all strains, but doubling dose varied from 0.4–1.0 (mean 0.6)

[a] Dubrova *et al.* (*63*).
[b] Sadamoto *et al.* (*64*).
[c] Fan *et al.* (*67*).
[d] Niwa *et al.* (*70*).
[e] Dubrova *et al.* (*71*).
[f] Barber *et al.* (*41*).
[g] Dubrova *et al.* (*40*).
[h] Niwa and Kominami (*66*).
[i] Barber *et al.* (*42*).
[j] Dubrova (*38*).

trons was 2.6 [for spermatid irradiation (*70*)] or 3.4 [for spermatogonial stem cells (*40*)].

Generally, using conventional mutation measurements, the risk of genetic effects of sparsely ionizing radiations for both somatic and germ cells has been found to decrease with reduction of dose rate down to ~10 mGy/min, attributable to more effective DNA repair at lower dose rates (*72*). However, for the induction of ESTR mutation in spermatogonial stem cells, radiation delivered over 100 h at a dose rate of 0.166 mGy/min was found to be as effective as that given at a rate of 0.5 Gy/min (*40*). Somers and coworkers found a twofold increase in mutation rate for spermatogonial stem cells after a γ-ray dose of 1 Gy, but when the mice were pretreated with 0.1 Gy 24 h before 1 Gy γ rays, they showed no increase in mutation rate (*73*). Despite the very high mutation rate found for ESTR sequences, estimates of the doubling dose (the dose at which the mutation rate is increased to twice the naturally occurring rate) are little different from those found for conventional mutation analyses in mice (*72*). Thus Dubrova and colleagues estimated the doubling dose to be around 0.5 Gy for pre-meiotic stage irradiation and 0.33 Gy in their later studies (*63, 68, 71*). Estimations were also made by Niwa and colleagues, but the doubling dose of 0.8 Gy for spermatid irradiation was the only value based on significant data (*67*), while Somers *et al.* found a value of 0.26 Gy after γ irradiation of pre-meiotic cells in outbred Swiss-Webster mice (*73*). Most recently, Dubrova (*38*) has estimated X-ray doubling doses for pre-meiotic cells varying between 0.4 and 1.0 in five different strains of mice; this

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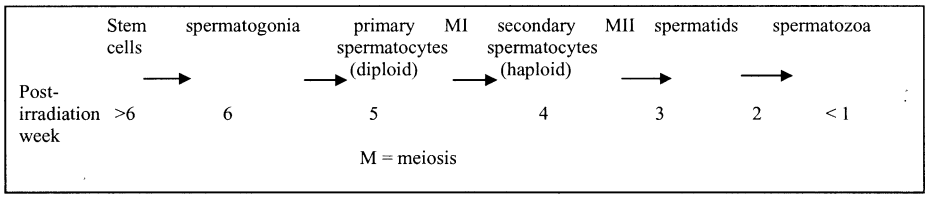


FIG. 1. Stages of mouse male germ cell development and approximate postirradiation mating times that reveal effects on specific stages.

variation was not due to differences in mutation induction in the different strains (see below) but rather to variation in spontaneous mutation rates. Standard errors on doubling doses are close to 50% (*38*) based on the assumption of a linear dose response.

The Controversy over the Mutability of Different Mouse Germ Cell Stages

In these ESTR mutation experiments, male mice were irradiated and mated at different times afterward to unirradiated female mice; depending on the time of mating, the effects of radiation on different stages of sperm development could be assessed. The longer the period between irradiation and mating, the earlier the developmental stage assessed (see Fig. 1). For example, offspring born to females mated to irradiated males at 3 weeks after irradiation would be derived from irradiated haploid post-meiotic cells (termed spermatids), whereas those mated at 6 weeks after irradiation are derived from irradiated diploid pre-meiotic cells (termed spermatogonia) (*74*). In conventional mutation experiments, these germ cell stages have shown differences in their mutation frequency for a given dose, with the highest frequencies generally found for sperm cells undergoing meiosis (spermatocytes) and for post-meiotic spermatids. For example, in a very large experiment using 3 Gy X rays, Russell *et al.* found a peak in mutability at 3–4 weeks postirradiation for both dominant visible and recessive lethal mutations (*75*).

Germ cell stage specificity for ESTR mutation was first reported by Niwa and colleagues (*64, 67*), after irradiating C3H/HeN mice with up to 3 Gy γ rays followed by mating to C57BL/6N females either within 1 week (assessing spermatozoa), at 2–3 weeks (spermatids), or at 10–11 weeks (stem cells). Only spermatid irradiation yielded significantly increased mutation rates. Similar data were published by this group for neutron irradiation; although they did report a significantly increased mutation rate for spermatogonial stem cells, this rate was lower than for spermatids (*70*). These ESTR data therefore tended to agree broadly with data on stage specificity using conventional gene mutation measurements.

However, in a parallel series of experiments by Dubrova and colleagues, the germ cell stage at which there was maximum sensitivity was found to be different (*40, 71*). They irradiated CBA/H mice with up to 1 Gy X rays or up to 0.5 Gy fission neutrons and found that the ESTR mutation rate was highest for pre-meiotic stem cells (6-week matings) and spermatogonial stem cells (10-week matings), whereas no increase was found for spermatid irradiations (3-week matings). Similarly, Somers and coworkers found that γ irradiation of spermatogonial stem cells (9-week matings) in outbred Swiss-Webster mice yielded a twofold increase in mutation rate, although no dose dependence was evident (*73*). In a further study, however, Dubrova *et al.* estimated the ESTR mutation rate after matings at weeks 3, 4, 5 and 6 and found that significant increases occurred at weeks 4, 5 and 6, indicating that spermatocytes were also responsive to radiation (*41*).

There are at least two possible reasons for this discrepancy in cell-stage sensitivity: differences in the mouse strain used and uncertainties in the timing of germ cell stages after irradiation. Although a large range of strains has not been tested, it is noteworthy that recent studies with up to five commonly used mouse strains showed similar ESTR mutation induction patterns in pre-meiotic germ cells (*38, 42*). Problems in assessing the germ cell stage may apply particularly to the spermatid stage, where rapid changes in the ability of germ cells to repair DNA damage occur. Early spermatids retain a capacity to repair DNA double-strand breaks, but as the spermatid matures, it undergoes a number of changes that may alter its sensitivity to damage. In late spermatids, the cell nucleus has condensed and DNA repair capacity ceases; this stage has been shown to be highly sensitive to various chemical mutagens, and this was partly explained by damage to DNA-binding proteins (protamines) involved in DNA compaction (*76*). Loss of protamine in mice has been found to cause severe DNA damage during condensation and packaging of DNA in the sperm head (*77*). Experiments designed to re-examine the consequences of irradiation of well-defined spermatid stages may help to resolve these discrepancies.

A further difficulty in interpreting the results of these experiments is uncertainty in the criteria used to score mutations. Mutations have been detected mostly by Southern analysis, and they are defined as DNA fragments of lengths that cannot be attributed to either parent, indicating that a minimum length difference needs to be defined to identify mutations. A shift of >2% of the distance between the parental bands (equivalent to 40–200 bp) was used by Niwa and coworkers (*64, 67*), but it is unclear whether a similar criterion has been used in other laboratories. However, use of a different method for assessing ESTR mutations, am-

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plifying single DNA molecules by PCR, showed that sperm derived from 1 Gy-irradiated stem cells had a significant 1.7-fold induction of *Ms6-hm* mutations (*39*). In this study, a shift of >2 repeat units was used as the criterion for mutation, but the same result was obtained by counting only those mutations displaying a shift of >5 repeat units. Although PCR amplification methods have to be used with care, single-molecule analysis has the advantage that it does not require mating to provide progeny for analysis. Additionally, in some studies the extent to which somatic mutations (as revealed by mosaicism) are included in the scores is not clear. Niwa (*69*) points out that the discrepancies may be due to different end points being measured in the two laboratories: embryonic instability after late-stage exposures and direct germline mutation after pre-meiotic exposures.

TRANSGENERATIONAL MUTATION STUDIES

In the context of cancers arising in the offspring of irradiated parents, mutation events occurring during parental germ cell development and mutations occurring in the cells of the descendent offspring might both play a role. Thus far in this review, mutations occurring in the first-generation offspring of irradiated parents have been considered. It is likely that many of these mutations occur during sperm cell maturation in the irradiated father (as shown in mice by small-pool PCR methods) and thus are not transgenerational in the strict sense. Mutations that are scored in the second-generation offspring of an irradiated parent can be classified unambiguously as transgenerational. Studies of TRDL mutation and a number of specific gene mutation systems are available.

Transgenerational Mutation at Mouse ESTRs

Early indications of transgenerational effects were seen in the experiments of Niwa's group (*64, 67*) and Dubrova *et al.* (*40, 78*). These have been followed up more comprehensively by both groups. Niwa and Kominami showed that maternally derived repeat sequences were mutated at the same rate as the γ-irradiated paternally derived repeats (by twofold or more) when the offspring were derived from immediate matings (spermatozoa irradiated) but showed no such increase when derived from 15-week matings (stem cells irradiated) (*66*). The lack of mutation induction in maternally derived ESTR sequences after premeiotically irradiated germ cell matings had also been established in earlier studies (*71*).

Irradiation of male mice with fission neutrons (0.5 Gy) and mating to unirradiated females after 10 weeks (assessing stem cell irradiation) yielded an average sixfold increase in ESTR mutation rate in the germline of their non-exposed offspring. When male and female offspring were mated further to unirradiated mice, it was found that the ESTR mutation rate was still elevated by about sixfold in males and by about 3.5-fold in females (*40*). These next-generation increases were derived in part from mutations that arose after fertilization, early in the development of the next-generation germline (''mosaicism'' shown by the same mutation being present in more than one offspring from the same breeding pair), but single mutations were also significantly more frequent ($P = 0.044$) in male second-generation offspring (but not in females; $P = 0.13$). In more extensive experiments, the same workers demonstrated that the transgenerational ESTR mutation rate increased for two generations after both X irradiation (2 Gy) and neutron irradiation (0.4 Gy) of different strains of mice (*42*). These multivariable experiments are summarized in Table 5 (data for spermatogonial irradiation only) and show little or no reduction in the persistence of elevated ESTR mutation rate even after two generations, irrespective of mouse strain or radiation type.

In addition to the data given in Table 5, CBA/H mice were tested for increases in mutation rate in the generation 1 and 2 progeny formed from spermatids irradiated in generation 0. Strikingly, in contrast to previous results for spermatid irradiation (generation 0) from these workers, both premeiotic and postmeiotic cells yielded similar mutation rate increases in these later generations (*42*). The increased mutation rate was transmitted equally through male and female lineages, suggesting that the signal inducing instability persists for at least two generations and is likely to result from an epigenetic modification of DNA.

TABLE 5
Radiation Induction of Transgenerational Mutation of ESTR Sequences (*42*)

Mouse strain	Radiation (dose)	Generation[a]	Mutation rate[b]	Rate ratio (to unirradiated)
CBA/H	None	—	0.07	—
	Neutrons (0.4 Gy)	0	0.21	2.9
		1	0.25	3.5
		2	0.20	2.7
	X rays (2 Gy)	0	0.19	2.6
		1	0.22	3.1
		2	0.24	3.3
C57BL/6	None	—	0.06	—
	Neutrons (0.4 Gy)	0	0.27	4.2
		1	0.16	2.4
		2	0.15	2.4
BALB/c	None	—	0.12	—
	X rays (2 Gy)	0	0.28	2.4
		1	0.31	2.6
		2	0.25	2.1

[a] Generation 0 comprises the offspring of the irradiated males, while generation 1 and 2 comprise the offspring of matings of generation 0 or generation 1 progeny to unirradiated partners, respectively.

[b] Calculated as the number of mutations/number of offspring per locus [since mutations in controls could originate from either parent, these frequencies are divided by a factor of 2 for comparisons with radiation-induced frequencies where it is assumed in the calculations that mutations originate from the male only; see ref. (*42*)].

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Transgenerational Mutation at Other Loci

These mouse transgenerational ESTR mutation studies find some support in studies of mutation at the pink-eyed unstable (p^{un}) locus in the mouse. The mouse p^{un} mutation was caused by the spontaneous disruption of the *pink-eyed dilute* locus on chromosome 7 resulting in a ~70-kb head-to-tail DNA sequence duplication. The mutation occurred in a C57BL/6J mouse, and most experiments continue to use this genetic background. The mutation leads to reduced pigment production in hair and retinal tissue, resulting in reduced coat and eye coloration. Spontaneous reversion is caused by deletion of one of the duplicated sequences and restores normal pigment production (*79*). The p^{un} locus is highly unstable; 1.8% or more of offspring are mosaic revertants (*80*). However, since reversion frequency is variable and can be as high as 12.2% (*81*), care must be taken in the interpretation of induced reversion frequencies. Reversion is generally detected as a somatic event, but germline reversion, which is highly stable and gives a phenotype indistinguishable from parental C57BL/6J mice, can also occur (*82*). Somatic reversion is proposed to proceed by homologous recombination (*79*).

Weak evidence indicative of transgenerational effects on p^{un} reversion after irradiation of male p^{un}/p^{un} mice has been reported (*83*). No significant elevation in fur spot frequency was reported; however, spots tended to be larger in irradiated mice. The authors of this study suggested that male germ cell irradiation can bring forward the time of p^{un} reversion in offspring and that there may be a transmissible elevation in reversion frequency. However, the data provided in this paper alone are insufficient by themselves to produce great confidence in this conclusion. Support for transgenerational mutation at this unstable locus comes from the study of p^{un} reversion in retinal pigment epithelium (RPE) after male germ cell irradiation (*84*). Assessment of reversion in RPE is considered to be more accurate than the fur spot assay (*85, 86*). Reversion was scored in p^{un}/p^{j} mice. The p^{j} allele carries a partial deletion of the *p* locus and does not revert to wild-type. Spontaneous reversion was, as expected, lower in p^{un}/p^{j} mice than in p^{un}/p^{un} individuals. X irradiation with 6 Gy at the stem cell stage (15 weeks prior to mating) had no effect on p^{un} reversion. However, spermatozoa-stage irradiation did induce significant reversion ($P < 0.01$, *t* test) in a dose-dependent fashion (doubling dose ~6 Gy or more). Most striking is the finding that irradiation of males carrying a p^{j} allele leads to reversion of maternally derived p^{un} alleles at a frequency indistinguishable from that due to irradiation of p^{un} carrying males. Thus untargeted events appear to occur and these events at least are truly transgenerational.

The Medaka fish (*Oryzias latipes*) has also been used for studies of transgenerational mutation using a specific locus test (e.g. *87*). Irradiated wild-type males are mated to tester stock (TS) females carrying a homozygous mutation (*wl*/*wl*) giving a white leukophore phenotype in embryos. Mutation of the male allele to *wl* will give rise to offspring with white leukophores; in the absence of mutation, leukophores are orange. The *wl* locus is probably of unusual structure because it has a high spontaneous mutation frequency; in this respect the locus may be analogous to p^{un} in the mouse. A γ-radiation dose-dependent induction of whole-body and mosaic *wl* mutants has been observed (*87*). Mosaics, which are indicative of a transgenerational mutation, were induced more readily than whole-body mutations. Most mosaics appeared to arise prior to the mid-blastula stage. Irradiation of males was at the spermatozoa/spermatid stages. The proportions of white/orange leukophores in mosaic mutants suggested that mutations occurred during development, i.e. were delayed. More recent studies (*88*) indicate that irradiation of Medaka sperm and spermatids, but not spermatogonia, can elevate mutations of an unirradiated maternal allele. This effect appears to depend on DNA damage being present in sperm DNA at the time of fertilization; no elevated mutation was detected in male F2 offspring from an irradiated male. These findings are similar to those of Shiriashi *et al.* (*84*) using the p^{un} RPE system.

Taken together, the mouse p^{un} and Medaka *wl* mutation data suggest that delayed and transgenerational mutation can occur at these loci. As with mouse ESTR studies, stage-of-irradiation differences and variable background mutation rates make interpretation difficult. Special structural features of p^{un} and probably *wl* are likely to influence the responsiveness of these loci to DNA-damaging agents.

Transgenerational Mutation in Humans

There are few data available from humans that address the issue of mutation in second-generation offspring of irradiated parents. The study of residents in the Semipalatinsk nuclear test site region does provide some information (*18*). In this study, no clear evidence of transgenerational effects at minisatellite loci over two generations was reported; effects were noted only in offspring of parents known to have been exposed to elevated radiation levels as a consequence of nuclear weapons tests. These results are insufficient to reach any general conclusion about transgenerational mutation in the human germline.

Considering all the transgenerational mutation data together leads us to the conclusion that genuinely transgenerational effects can be seen in some DNA sequences in the mouse and Medaka fish. However, evidence for transgenerational mutation in humans is at lacking present.

ENVIRONMENTAL MONITORING STUDIES

The majority of reports concerning radiation-associated changes in mutation rates at minisatellite and related loci concern humans and laboratory mice. However, a few papers in the literature consider other eukaryotic species. Sampling of organisms from radiation-contaminated areas

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TABLE 6
Summary of Chernobyl-Zone Molecular Mutation Studies

Species	Reference	Locus	Dose/dose rate	Effect	Comments
Laboratory mice: C57BL/6 and BALB/c	Wickliffe *et al.* (*93*)	Mitochondrial cytochrome b	Cumulative doses of 1.2–1.6 Gy at ~0.04 Gy/day	No significant elevation in mutation rate postexposure	Criticized on technical grounds by Dubrova (*94*)
Barn swallows (*Hirundo rustica*)	Ellegren *et al.* (*95*)	Microsatellites *HrU6, HrU9*	No dosimetry, difficulties with migration etc.	*HrU6*: higher (10-fold or more) mutation frequency in Chernobyl zone birds compared to local and distant (Italian) control zone birds. *HrU9*: mutation frequency same in Chernobyl and local control zone birds; both elevated (~2.5-fold) compared to distant control zone	Increased partial albinism and reduced fitness also reported for Chernobyl zone birds
Cultivated wheat (*Triticum sp.*)	Kovalchuk *et al.* (*96*)	13 single-copy microsatellites	Contaminated plots: 900 Ci km^{-2}, 0.3 Gy (2/3 external) control plots: <1 Ci km^{-2}[a]	~Sixfold evaluation in mutation rate in contaminated plot grown plants	Mutation rate in control plot grown plants also high, 1.03×10^{-3} per locust

[a] 33.3 TBq km^{-2} and 37 GBq km^{-2}, respectively.

provides a useful method for assessing the mutational risk associated with specific areas. This environmental monitoring approach has been used to examine the risks associated with living in the zone heavily contaminated as a consequence of the Chernobyl disaster.

Studies of this nature face two significant problems. First, accurate radiation dosimetry is usually very difficult to obtain, and consequently dose estimates are at best poor and at worst absent. Second, environments such as the Chernobyl site have also been contaminated with heavy metals and other pollutants as well as radiation (*89*). Studies of human populations in contaminated areas face similar problems. Thus environmental monitoring studies are unlikely to provide either unambiguous evidence of radiation causation or robust dose–response data. A related difficulty is that laboratory-generated reference data are rarely available for the species investigated. Minisatellite and ESTR mutations have been shown to be elevated in herring gulls (*Larus argentatis*) and mice exposed to pollutants other than radiation in highly industrialized regions (*90–92*). The exposure of laboratory mice to polluted and control environments followed by mating and analysis of ESTR mutation in pedigrees has proven to be of great value in determining the mutagenic potential of an industrial site (*91*). A follow-up study was conducted in which mice were housed in HEPA-filtered cages or nonfiltered cages at either the polluted or control sites (*92*). In this way it was demonstrated that particulates greater than 0.3 μm in diameter were the major contributor to elevated germline mutation frequencies (*92*).

Table 6 summarizes the published data available for environmental radiation exposure; all refer to studies examining mutations in organisms exposed to contaminated zones at Chernobyl. Taken together, these studies provide some evidence for Chernobyl-contaminated zones posing an elevated risk of mutation in the germline. This is best illustrated by the study of wheat in which a sixfold elevation in mutation rate was observed in plants grown on contaminated land (Table 6). Here it remains unclear whether the increases are due to radiation exposure or to other contaminants.

MECHANISMS OF TANDEM REPEAT DNA LOCUS MUTATION

Background

In the last few years there have been several reports of unusually high levels of genetic changes or unexpectedly persistent changes in cells or animals caused by radiation. Although the causal mechanisms underlying these changes are not fully understood, these findings have led to a rethinking of the way in which radiation acts in cells. Although much of the damage to cells is processed rapidly by cellular repair enzymes (i.e., within a few hours of irradiation), there is evidence that cellular responses also occur over much longer periods.

In a classic series of experiments in the early 1980s, Kennedy and Little studied how normal mammalian cells were transformed into cells that could form tumors and showed that radiation caused high-frequency events that predisposed the cells toward later (spontaneously occur-

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ring) changes (*97, 98*). In follow-up experiments, it was found that the mutation frequency in nonessential genes such as *HPRT* was persistently increased in a large fraction of clones surviving irradiation (*99*). The mutant frequency in individual clones was highly variable but sometimes exceeded 1 per 1000 cells. These delayed mutations appeared to be predominantly point mutations, while the direct action of radiation tends to yield predominantly larger genetic changes (*5*).

Chromosomal aberrations have also been found to continue to be formed for a prolonged period after irradiation. One-cell mouse embryos irradiated with X rays or neutrons showed an approximately linear increase in the frequency of chromosomal aberrations per cell in the first, second and third mitoses postirradiation. The relatively high frequency of aberrations, especially for neutrons, and the occurrence of chromatid-type aberrations on the third mitosis after irradiation suggested that new aberrations were being produced in postirradiation cell cycles (*100, 101*). Genetic instability in clones of cells surviving irradiation has also been described for α-particle irradiation of cultured hematopoietic stem cells from CBA/H mice. In this case, chromosomal aberrations were measured in cell clones surviving 3 Gy X rays or 0.25–1 Gy α particles (0.5 Gy from ^{238}Pu α particles corresponds to an average of one track per cell). About 50% of the clones surviving α-particle irradiation carried aberrations; these were mostly chromatid-type aberrations, suggesting that they had arisen many generations after irradiation (*102*).

Chromosomal instability in bone marrow cells was also found to be transmissible *in vivo* by transplanting male cells irradiated with α particles into female recipients (*103, 104*). The repopulated hemopoietic system showed instability that persisted for up to 1 year. Alpha particles have also been shown to induce similar delayed chromosomal effects in the bone marrow of two out of four normal humans (*105*). It was suggested that the lack of effect in some individuals reflects genetic determinants that vary in the human population, and additional studies of other inbred mouse strains have also been found to show varying levels of this form of genetic instability. It should be noted, however, that not all studies with mice or normal human fibroblasts have found evidence for the induction of transmissible chromosomal instability by radiation (*106–108*). In an attempt to link the delayed appearance of chromosomal aberrations in cultured mammary epithelial cells to cancer proneness, Ponnaiya *et al.* (*109*) measured this form of instability in strains of mice differing in their sensitivity to radiation-induced mammary cancer. Strikingly, cells from the more sensitive strain (BALB/c) showed a marked increase in the frequency of chromatid aberrations after 16 population doublings, while the less sensitive strain (C57BL/6) showed no increase in aberrations over the control level.

Possible mechanisms for these novel events include persistence of the damaging agent, persistence of certain forms of DNA damage or modification (e.g. methylation), the repair of damage leading to rearrangements of the genome, which themselves upset the correct functioning of the cell (e.g. ''position effects'' on blocks of genes), and the induction of long-lived changes in gene expression, such that enzymatic activities (e.g. DNA polymerases) involved in the fidelity of genome maintenance do not function properly. Some of these mechanisms may also lead to ''untargeted'' changes in the cell's genome; i.e., the site of radiation damage is not the site of the final genetic change.

Enhanced Mutation at ESTRs

The high levels of mutation at ESTRs display some similarities to the events described above, but it is not clear whether related mechanisms are involved. The events described in other sections of this review indicate that ESTR mutations occur at a radiation-induced frequency that is much higher (by about 100-fold) than could possibly be explained by the amount of DNA damage we know occurs after specific radiation doses. How then can this happen?

It must be assumed that some radiation-induced event triggers the ESTR sequences to become genetically less stable but that this primary event may not occur at these sequences. Well-documented pathways leading to mutation involve misreplication during DNA synthesis or aberrant recombination of sequences. If either of these pathways is involved, then we must invoke an efficient mechanism for transmitting signals from initial sites of damage to ESTR sequences and/or suggest that the ESTR sequences are more prone to mutational changes than other sequences.

It seems unlikely that the ESTR mutations can be attributed to radiation damage at another single gene whose product can influence changes in the ESTR DNA: The frequency of mutations at ESTRs would still be governed by the frequency of the initial mutation event and the number of genes involved. However, we must bear in mind that complex mechanisms govern the maintenance of DNA. Each human cell contains about 2 m of DNA that needs to be tightly packaged into chromatin so as to fit into the cell. While the DNA has to be packaged precisely, access must be provided to ensure that the cell can carry out its functions (e.g. to retrieve genetic information from its genes, replicate DNA when required to do so, and ensure genetic stability). Accessibility of the DNA in chromatin is governed by chromatin remodeling factors, including proteins that can covalently modify histones by the addition or removal of acetyl, phosphate or methyl groups (*110*). These histone modifications change the extent to which DNA is bound to the nucleosomes, influencing the ability of exogenous agents to damage DNA (*111*) and the ability of damage-response proteins to access DNA. Specific combinations of the various chromatin remodeling factors, making up a pattern or ''code'', are considered to be responsible for controlling access to specific regions of the genome.

In this context, it could be that access to ESTRs is suppressed by a specific histone code and that changes to this

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code render the ESTRs much more sensitive to mutagenic events. This would mean that a mutation in any one of a number of genes involved in maintaining the code would influence events in a non-targeted fashion [e.g. genes encoding acetylases, deacetylases, kinases (which add phosphate groups), phosphatases (which remove phosphate groups), methylases and demethylases, etc.]. It is also possible that the chromatin code for highly repetitive sequences differs between mouse and human. In mice, ESTR sequences are distributed throughout the genome, whereas in humans, minisatellites occur mainly in the subtelomeric regions of chromosomes. This difference in distribution could influence how ESTRs are metabolized in mice and humans, since the chromatin code in the respective regions where the ESTRs reside may well be different.

A number of authors have suggested that epigenetic changes may be involved in transgenerational ESTR mutation (*40, 42, 69*). Epigenetic modifications will alter DNA function without changing the underlying nucleotide sequence; one example is that of genes that can be silenced through the methylation of CpG dinucleotides in their regulatory sequences. Until recently, it had been thought that DNA modifications were erased during mammalian gametogenesis, so that their ability to be transmitted was in doubt. The transgenerational inheritance of epigenetic modifications has now been demonstrated for certain alleles (variant forms) of mouse genes: It was found first for alleles of the *Agouti* coat color gene (*112*), then for an allele of the *Axin* gene involved in embryonic axis formation (*113*). In both examples, the alleles were associated with retrotransposon insertions, and their expression was correlated with differential DNA methylation. In the case of the *Axin* gene, transgenerational inheritance was found after both paternal and maternal transmission, while it was found only after maternal transmission for the *Agouti* gene. Some evidence was also provided for differences between mouse strains in their ability to erase these epigenetic modifications after fertilization.

In the development of human cancers, it is known that methylation of tumor suppressor genes provides a mechanism for the somatic loss of gene expression (''epimutation''), but recently individuals with multiple cancers have been found with a germline epimutation of the DNA mismatch repair gene *MLH1* (*114*). These heritable epimutations were the result of persistent hypermethylation of the promoter region of the *MLH1* gene, again consistent with incomplete erasure of DNA methylations in the germline.

How Can We Unravel What is Happening?

The isolation of mutant organisms that show altered levels of response has been a very valuable means of understanding how damage-response pathways operate. Characterization of these mutants ultimately leads to identification of the gene(s) that is altered to give the difference in response. Once a gene is identified and its DNA sequence determined, clues are often provided that make it possible to determine the function of the gene product (an enzyme or a structural protein) in the cell. Other components in the pathway can be found, for example, using modern biochemical approaches such as immunoprecipitation and mass spectrometry to show which proteins interact with a known protein in the pathway.

Mutants may be used from a variety of organisms, ranging from unicellular bacteria and yeasts, where it is much easier and quicker to identify and analyze mutants, through to mammals such as the mouse, which are more likely to mimic the situation in humans. In the case of minisatellite mutations, some insights have already been obtained with yeasts. Yeast strains carrying the unstable human minisatellite *CEB1* (repeat unit about 40 bp) showed losses and gains of repeat units in meiosis, and this instability was found to require the activity of genes involved in the formation of DNA double-strand breaks (*115*). Similar data were obtained for trinucleotide repeats in yeast (*116*). Recently, the *HRAS* minisatellite (28-bp repeat unit) was found to stimulate double-strand break formation during yeast meiosis; the *RAD1* gene encoding a nuclease important in both excision and recombination repair pathways was shown to be required for expansion but not contraction of repeats (*117*). Further, while minisatellites are normally stable during mitotic growth in both yeast and humans, the stability of the *CEB1* minisatellite was compromised during mitotic growth of yeast strains defective for genes involved in DNA replication (specifically nucleases removing ''flaps'' of sequence generated in lagging strand synthesis) (*118*). All together, these data indicate that minisatellites in yeast may mimic several of the characteristics of human minisatellites, that DNA breaks appear to be required to generate instability, and that nucleases involved in removing additional sequence generated in DNA repair or replication are important to protect against instability. These findings do not suggest an exclusive model for minisatellite instability, although from the models put forward to date, it seems likely that homologous recombination is involved at some stage of the process (*117, 118*). In fact, the data imply that mutations could arise through several mechanisms, depending on whether there is loss or gain of DNA or changes in DNA sequence.

We have summarized above the evidence for the transgenerational transmission of a hypermutable state at ESTR loci in mice. It is worth noting that some years ago Fabre and Roman (*119*) showed that homologous recombination could be induced in an unirradiated yeast cell by fusing it to an irradiated cell. This process could be said to mimic, in somatic cells, the fusion of irradiated sperm and nonirradiated egg in the mouse experiments. Following ideas initially proposed by Holliday (*120*), Fabre and Roman suggested that radiation caused the release of some factor(s) that normally represses recombination. Fabre followed this up with further (unpublished) data describing a radiation-induced recombination mechanism that is transmissible

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through mitotic division in yeast. These events in yeast have much in common with ESTR mutations in mice: Recombination was seen in a high proportion of cells, and it could not be explained by a targeted event.

Direct tests for a link between homologous recombination processes and ESTR mutation in mice have not yet been made. However, recombination is commonly associated with the visible crossing over of chromosomes during meiosis, and Barber *et al.* (*41*) compared crossover frequency with germline ESTR mutation frequency after 1 Gy X rays. They found an approximately threefold increase in ESTR mutations but no general change in crossover frequencies measured at 25 sites on six different chromosomes. This shows that there is no general correlation between mutation and crossing over, although it is difficult to test whether elevated recombination may occur at specific stages of spermatogenesis or whether ESTR mutation sites may differ in sensitivity from the tested sites (see above).

It is also of mechanistic importance to know at which stage of mouse development the ESTR mutations occur. The finding of mutations in maternal alleles of offspring after irradiation of male germ cells (*42, 66*) suggests that the mutational mechanism operates in the zygote after fertilization, where paternal and maternal nuclei share a common environment. Interestingly, a trans-acting process has been found in mice in which a reporter gene was activated upon fertilization by irradiated sperm (*121*). However, it has also been shown by direct analysis of mutation in spermatozoa of irradiated adult males that ESTR mutations can be detected at 10 weeks after irradiation (*39*). Taken together, these data suggest that some radiation-induced mutations occur directly in the germ cells, while other mutations occur later due to instability; it is possible that induction mechanisms differ between these two stages.

POTENTIAL IMPLICATIONS OF MINISATELLITE MUTATION FOR HUMAN HEALTH

Minisatellite Expansion and Contraction in Human Disease

Since minisatellite mutation events occur both in the germline and in somatic tissues, they have been studied in both inherited disease and cancer. They appear to be associated with genetic disease by virtue of the ability of minisatellite alleles of different lengths to alter gene coding sequences (*28*), influence gene expression (*122, 123*), or generate fragile sites (*124*). For example, a minisatellite upstream of the cystatin B (*CSTB*) gene is normally present in two or three copies but is expanded to 30–80 copies in individuals with a specific form of epilepsy (*125, 126*). The expanded alleles exhibit a greatly increased mutation rate (47%) (*127*) and give rise to a dramatically reduced level of *CSTB* gene expression (*128*).

The *INS* minisatellite has been shown to influence the transcription of both the *INS* gene (*129*) and the insulin-like growth factor 2 (*IGF2*) gene (*130*), and certain genotypes may be associated with a number of different conditions/phenotypes including insulin-dependent diabetes (*131, 132*), insulin sensitivity and secretion (*133*), childhood obesity (*134*), birth size (*135*) and polycystic ovary syndrome (*136*).

Homozygosity for a particular allele (L) of the MNS16A minisatellite, located downstream of the human telomerase (*TERT*) gene, has been associated with a twofold increased risk of lung cancer (*137*), whereas rare alleles of the *HRAS* minisatellite may be associated with increased risk of a variety of cancers including leukemia (*138*), lung (*139*), breast (*140*), brain (*141*), colorectal (*142*), ovarian (*143*) and bladder (*138*). However, their role as a risk factor in tumorigenesis is still somewhat controversial (*144*). Some doubt has recently been cast on the conclusions drawn by the original association studies. While the mutation rate of the *HRAS* minisatellite is much lower than previously estimated, the ''rare alleles'' noted in European populations have been found to predominate in Africans (*144*). The proposed association between rare alleles of the *HRAS* VNTR and various cancers therefore remains to be confirmed or refuted by additional studies. Rare alleles of the *HRAS* minisatellite have also been noted in tissue from spontaneously aborted human embryos (*145*), but the results of this study are open to question owing to the lack of proper controls.

A number of other genes possess minisatellite alleles that have been claimed to display an association with a particular disease or trait [reviewed by Nakamura *et al.* (*146*)]. It must be realized, however, that in the absence of *in vitro* studies that provide evidence for a direct effect on gene expression or function, all these associations may simply represent linkage disequilibrium between a given minisatellite allele and the actual pathological lesion. In other words, the minisatellite allele in question can be detected at high frequency in individuals with the disease, but its presence does not necessarily cause the disease.

Minisatellites often contain a sequence with strong homology to the bacterial Chi (χ) element, which is a recombination signal in bacteria. Minisatellites have been reported as hotspots for homologous recombination in human cells (*147*) and are also capable of stimulating homologous recombination *in vitro* (*148*). However, minisatellite instability may not be an intrinsic property of the tandemly repetitive DNA but may rather be directed in some way by elements external to it. Thus the alternative view holds that minisatellites may have evolved as ''by-products of localized meiotic recombination [hotspots] in the human genome'' (*25*).

Minisatellite core sequences have been reported in association not only with oncogenic chromosomal translocation breakpoints (*149–153*) but also with the breakpoints of gross deletions causing inherited disease (*154–156*). In a recent meta-analysis of translocation breakpoints in human inherited disease and cancer, the minisatellite core element

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was found to be significantly over-represented at such sites (*157*). These sequences may represent a distinct class of recognition element for V(D)J recombinase that plays a key role in the generation of antibody diversity for the immune system (*158*).

It should be mentioned that we have no knowledge as to whether minisatellite mutations play any role in determining pregnancy outcomes, nor have their potential roles in the developmental process been investigated. These may well be worthwhile areas of investigation.

Finally, there are some reports of sequence similarities between human minisatellites and viral sequences that could potentially be of some importance. Thus a minisatellite termed TBE (containing between 2 and 19 copies of a 15-bp repeat) has been found to be homologous to sequences of type D retrovirus (*159*). In the same vein, another human minisatellite (MEB-1) is similar to a coding sequence within the Epstein-Barr virus genome, with both sequences resembling the bacterial χ element (*160*).

CONCLUSIONS

TRDL mutation has been used as a biomarker of human radiation exposure with mixed success. TRDL mutations have been seen in residents of regions contaminated after the Chernobyl accident and other human populations exposed predominantly to internal emitters. However, studies with predominantly external radiation exposure (e.g. Chernobyl liquidators, radiotherapy patients, A-bomb survivors) are largely negative, although studies of the Semipalatinsk population are positive. The interpretation of these studies has been hampered by small sample sizes and inadequate radiation dosimetry. It is notable that the positive studies generally involve low-dose exposures with significant contributions from internal emitters.

Studies of ESTR mutation suggest that TRDL mutation can be used as a biomarker of radiation exposure in the mouse. Inconsistencies in the published data regarding germ cell stage sensitivity are, however, difficult to resolve. Overall, despite a lack of clear dose–response data, radiation doubling dose estimates are broadly consistent with those currently in use for estimating germline mutational risk. It is likely that many of the radiation-induced mutations occur distant to direct DNA damage targets. Studies in the mouse additionally suggest that transgenerational mutation can occur at ESTR loci. Evidence indicative of transgenerational mutation in humans is lacking at present.

Studies of the mechanisms of TRDL mutation are limited to mouse ESTRs, and the mechanisms of mouse ESTR mutation may be distinct from those of human minisatellite mutation. A number of feasible approaches to improving the understanding of ESTR mutation were outlined earlier.

It is clear that some minisatellite variants are associated with human disease and may affect critical processes such as transcription or mRNA splicing. However, caution is required, since little direct evidence links minisatellite variation to disease and no specific health effects have been demonstrated to occur as a consequence of radiation-induced minisatellite mutations. Thus no generalizations of potential health effects of TRDL mutation should be made at present. Despite our lack of knowledge regarding the implications of TRDL mutations and their origin, from the epidemiological data it is highly improbable that the newer TRDL data will result in a dramatic revision of germline risk estimates. For cancer and other conditions, however, these mutations may play a role, and only further research will determine whether this is the case. In addition, we have no knowledge as to whether TRDL mutations might influence pregnancy outcome, development or viral disease. A full knowledge of the number, diversity and evolutionary conservation of minisatellites and ESTRs is not yet available; these areas deserve further research effort. Hence it is evident that there are many uncertainties and gaps in our knowledge concerning the roles of TRDL mutation in human disease; these will be clarified only by further research into the mechanisms and consequences of this class of mutation.

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