Human minisatellite mutation rate after the Chernobyl accident

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Germline mutation at human minisatellite loci has been studied among children born in heavily polluted areas of the Mogilev district of Belarus after the Chernobyl accident and in a control population. The frequency of mutation was found to be twice as high in the exposed families as in the control group. Mutation rate in the Mogilev families was correlated with the level of caesium-137 surface contamination, consistent with radiation induction of germline mutation.

THE accident on 26 April 1986 at reactor 4 of the Chernobyl nuclear power station resulted in the largest reported accidental release of radioactive material[1]. Many regions within the European part of the former Soviet Union were heavily contaminated by radioactive fallout. In the first three months after the accident, acute irradiation of humans occurred through external and internal exposure to iodine-131 with a half-life of 8 days[2]. Following ^{131}I decay, exposure to more stable isotopes, mainly ^{137}Cs, became the main source of radiation risk for people in contaminated regions.

Despite the magnitude of the Chernobyl disaster, little is known about the effects of radiation exposure in human populations inhabiting polluted areas. A considerable increase of thyroid carcinoma in children around Chernobyl has been reported[3,4], as well as an elevated frequency of chromosome aberrations in most residents tested[5]. Additionally, the frequency of congenital malformations in newborns and human embryos has increased in heavily contaminated areas of Belarus following the accident[6,7]. In contrast, nothing is known about the effects of chronic radiation exposure on germline mutation in humans.

Tandem repeat minisatellite loci have several potential advantages for monitoring germline mutations in humans. The very high rate of spontaneous mutation altering allele length (repeat copy number)[8–10] provides a system capable in principle of detecting induced mutations in relatively small population samples. We have previously shown that acute doses of ionizing γ-radiation cause a significant increase in minisatellite germline mutation rate in mice[11], detectable by DNA fingerprint analysis of small numbers of families at doses substantially lower than can be monitored by standard genetic techniques. A similar increase in mutation rate at one unstable minisatellite locus has been found in the progeny of irradiated mice[12,13]. Here we report that minisatellite mutation rate is also unusually high in exposed human populations following the Chernobyl accident.

Population variability and mutation scoring

Blood samples were collected from 79 families (father, mother, child) inhabiting the heavily polluted rural areas of the Mogilev district of Belarus (Bychoskii, Krasnopol'skii and Cherikovskii regions; Fig. 1). This cohort is composed of children born between February and September 1994 for whom both parents were continuously resident in the Mogilev district from the time of Chernobyl accident. The control sample consists of 105 non-irradiated caucasian families sex matched to the exposed group of offspring. Because the entire Belarus area was contaminated, and it proved logistically difficult to sample Belarus families with children born before the Chernobyl accident, the control group was from the United Kingdom.

DNA fingerprints were produced from all families by using multilocus minisatellite probe 33.15 (ref. 8) and two hypervariable single-locus minisatellite probes MS1 and MS31 (loci *D1S7*, *D7S21*)[9]. In addition, most families were DNA profiled with the minisatellite probes MS32 and CEB1 (loci *D1S8*, *D2S90*)[9,14]. These probes, chosen for their relatively high mutation rates[9,10,14], provided sufficient information to verify the parentage of all children analysed, even in the presence of mutation[10]. Mutants were identified as novel DNA fragments present in the offspring that could not be ascribed to either parent. DNA fingerprints were only scored over the well-resolved region (3.5–22 kilobases).

Examples of families with an offspring containing a mutant band are shown in Fig. 2. Using probe 33.15, 18.12 ± 0.36 (s.d. = 5.58) DNA fingerprint bands were scored per offspring in the control group and 17.52 ± 0.35 (s.d. = 3.24) bands in the offspring of irradiated parents ($t = 1.18, P > 0.05$; Bartlett test for homogeneity of group variances, $\chi^2 = 0.89$, d.f. = 1, $P > 0.05$). Furthermore, the frequency of band sharing between parents in the control and Mogilev samples was indistinguishable

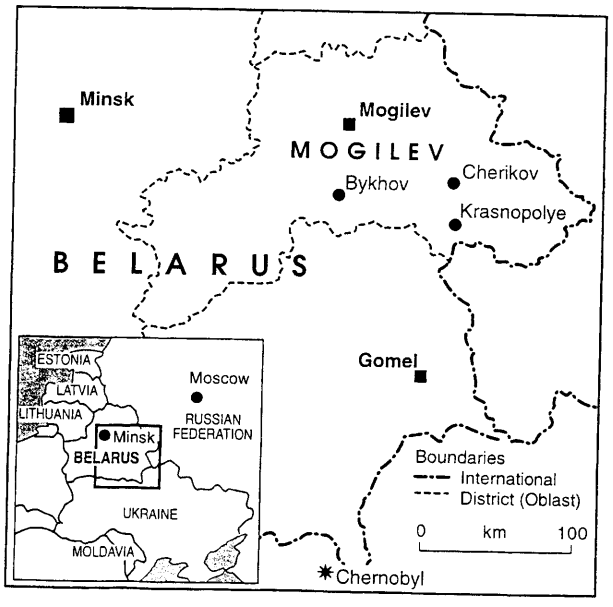

FIG. 1 Map showing the study area.

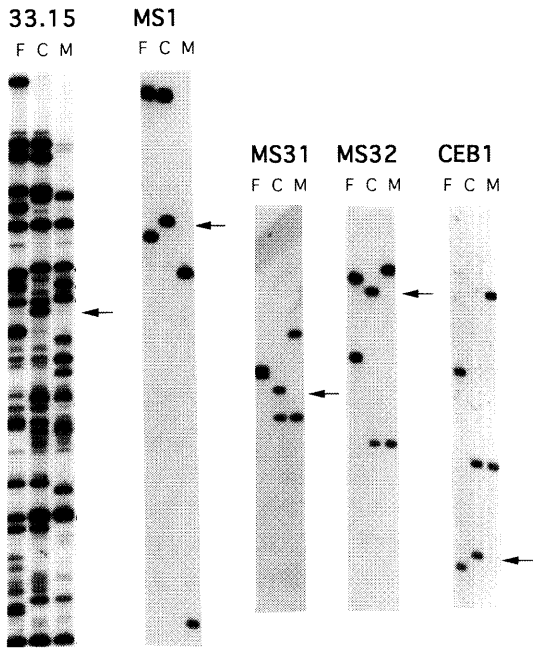

FIG. 2 Examples of minisatellite germline mutation. DNA profiles were produced for each father (F), child (C) and mother (M) using probes 33.15, MS1, MS31, MS32 and CEB1. New mutant bands are arrowed.
METHODS. Samples (4 μg) of DNA extracted from blood were digested to completion with *Alu*I, electrophoresed through a 35-cm (for probe 33.15) or 40-cm (for single-locus probes) long agarose gel (SeaKem, type LE, FMC) in 1 × TBE buffer (89 mM Tris-borate, pH 8.3, 2 mM EDTA), transferred to a nylon membrane (Hybond-Nfp, Amersham) and hybridized to ^{32}P-labelled probes as described elsewhere[28]. All autoradiographs were scored by eye by three independent assessors. New mutant bands were identified as offspring bands present in neither parent.

(0.124 ± 0.007 and 0.118 ± 0.008, respectively, $t = 0.57$, $P > 0.05$). Parental allele sizes and allele-length frequency distributions were also determined for the four single loci tested (Fig. 3). For three loci, allele frequency distributions were indistinguishable in the control and irradiated populations; for MS32, minor but significant differences between the two groups were found. We therefore conclude that any differences in minisatellite variability between these two caucasian populations are likely to be negligible.

Mutation rate

Spontaneous mutation rates in the control families were similar to those previously measured in caucasian populations using these five probes[9,10,14] (Table 1). In contrast, the frequency of mutant bands was elevated in the offspring of irradiated parents. By using multilocus probe 33.15, we found a statistically significant twofold increase in mutation frequency in the offspring of irradiated parents. Reprobing with four single-locus probes showed that some mutant bands detected by probe 33.15 are derived from minisatellites MS1 and MS31, whereas probes MS32 and CEB1 detect sets of bands that do not hybridize with 33.15. We therefore divided mutants scored by 33.15 into those attributable to MS1 plus MS31, and to those from other unknown loci (Table 1). An increased mutation rate in the exposed group was found for both sets of loci. A statistically significant doubling of mutation rate in Mogilev sample was also found at the highly unstable locus *D2S90* (probe CEB1). In contrast, a decrease of mutation rate in the exposed group was found at locus *D1S8* (probe MS32). However, very few mutants were found in either group, and the data do not deviate significantly from those expected for equal mutation rate in both populations (see Table 1) or for a doubled rate in the Mogilev group (Poisson approximation, $P = 0.11$). Finally, we estimated the total frequency of mutant bands in offspring analysed using all three independent probes (33.15, MS32, CEB1); the overall mutation rate was again twofold higher in the Mogilev group.

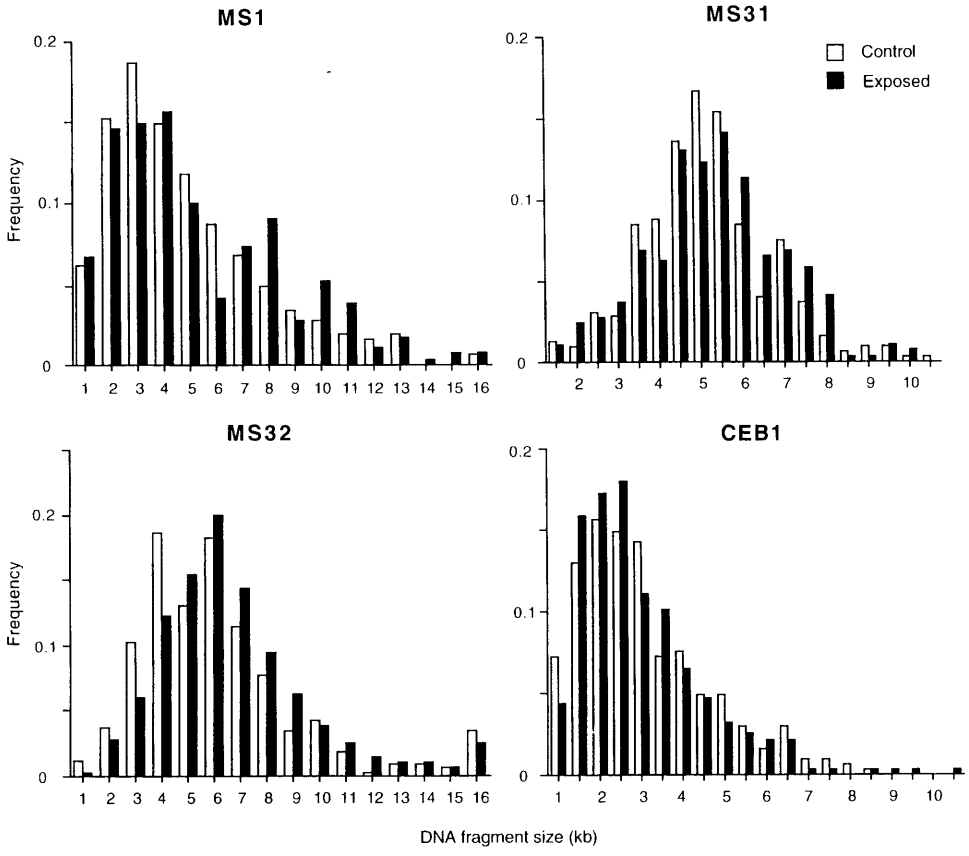

FIG. 3 Distribution of minisatellite allele sizes. DNA fragment sizes were estimated by the method of Southern[29], using a 1-kb DNA ladder (GIBCO, BRL) included on all gels, and binned into 0.5-kb (MS31, CEB1) or 1-kb (MS1, MS32) intervals. Differences between the control (white bars) and exposed (black bars) population distributions were analysed using the Kolmogorov–Smirnov two-sample test: MS1, $P > 0.05$; MS31, $P > 0.05$; MS32, $P < 0.05$; CEB1, $P > 0.05$.

TABLE 1 Mutation rates in control and exposed populations

Probe	Locus	Control group: Total no. of bands in offspring	Control group: No. of mutations	Control group: Mutation rate per band	Exposed group: Total no. of bands in offspring	Exposed group: No. of mutations	Exposed group: Mutation rate per band	Ratio exposed to control	P*
33.15:		1,903	19	0.0100	1,384	28	0.0202	2.03	0.0126
-MS1 + MS31†	*D1S7, D7S21*	387	9 (7 + 2)‡	0.0233	281	11 (7 + 4)‡	0.0392	1.68	0.1794
-other bands		1,516	10	0.0066	1,103	17	0.0154	2.34	0.0288
MS32†	*D1S8*	164	3	0.0183	150	1	0.0067	0.36	0.5449
CEB1	*D2S90*	164	11	0.0671	150	20	0.1333	1.99	0.0446
33.15 + MS32 + CEB1§		1,491	23	0.0154	1,615	49	0.0303	1.97	0.0041

* Probability using Fisher's exact test of independence (two-tailed).

† All MS31 and MS32 mutants, including four showing a single repeat unit change, were verified by MVR–PCR analysis of progenitor and mutant alleles[17,18].

‡ Number of mutants scored by MS1 and MS31 are shown in brackets.

§ Data presented for the families studied by all three probes.

Blood samples from the control group were collected in the United Kingdom, and it is possible that the increased mutation rate in the Mogilev group might reflect intrinsic differences in minisatellite instability between these two caucasian populations. Indeed, profound allelic differences *in cis* in mutation rate at MS32 have already been found particularly among Africans, where they influence allelic variability[15] yet have little effect on population mutation rate as assayed here. However, increased mutation rate was seen with three groups of independent minisatellites (MS1 + MS31, other bands scored by probe 33.15, and CEB1). Further, very similar increases in mutation rate (1.7–2.3-fold) were found in the exposed group for each of these minisatellite systems, indicating that increased mutation rate cannot be attributed to a single locus that has accumulated unusually unstable alleles in the Mogilev population. We therefore conclude that the difference in mutation rate found between the two groups of families is probably caused by environmental, rather than intrinsic genetic, factors. Environmental mutagens might include industrial or agricultural pollutants as well as post-Chernobyl radioactive contamination.

Surface contamination and mutation rate

Although the exact radiation dose received by each person in the Mogilev sample is not known, the level of surface contamination by ^{137}Cs provides a reasonable indicator of collective doses. A significant correlation between the total radioactive fallout after the Chernobyl accident and the level of ^{137}Cs contamination was found for most regions of Belarus[16]. Families were therefore divided according to median ^{137}Cs surface contamination (Fig. 4*a*) into those inhabiting less contaminated (surface contamination $< 6.8\,\mathrm{Ci\,km^{-2}}$) and more contaminated ($> 6.8\,\mathrm{Ci\,km^{-2}}$) areas. The total mutation rate (probes 33.15 + CEB1 + MS32) in more-contaminated areas was 1.5 times higher than in less-contaminated areas (0.0390 versus 0.0259 per offspring band, $P = 0.0413$, Fisher's exact test of independence, one-tailed), and both were higher than in the unexposed population (0.0154 per offspring band, $P < 0.05$ for both comparisons; Fig. 4*b*). The frequency of observed mutations was greater in the more highly exposed families both for 33.15 and for CEB1 + MS32 (data not shown). This correlation of mutation rate within the exposed group with surface contamination levels is consistent with the possibility that the increased frequency of minisatellite mutations found in the exposed group is a direct consequence of irradiation following the Chernobyl accident. It is possible, however, that other non-radioactive contaminants from Chernobyl, such as heavy metals, could be responsible for the observed, apparently dose-dependent increase in mutation rate.

Mutational spectrum

At the four single loci tested, 55 mutants were found, with most mutations at CEB1. The parental origin and germline length change were defined for each mutant band. The ratio between male and female germline mutation rate is similar in both groups (18 paternal versus 5 maternal mutations in the control group, and 28 paternal versus 4 maternal in the exposed group; $\chi^2 = 0.78$, d.f. = 1, $P > 0.05$). The incidence of mutations involving gain or loss of repeat units is similar in both groups (14 gains versus 9 losses in the control group, and 17 gains versus 15 losses in the offspring of irradiated parents; $\chi^2 = 0.32$, d.f. = 1, $P > 0.05$). Most mutation events involve the gain or loss of only a few repeat units, and the size distributions of mutations are not distinguishable between the two groups (Fig. 5*a*). The sizes of progenitor alleles are also similar in both groups (Fig. 5*b*). Mutants at MS31 and MS32 (all paternal) in the control and irradiated families were further characterized by minisatellite variant repeat mapping by polymerase chain reaction (MVR–PCR)[17,18] to determine the order of variant repeat units along progenitor and mutant alleles (data not shown). Most mutants in both groups showed features expected from previous analyses of mutants identified in pedigrees and by single-sperm analysis[18], namely mutational polarity with changes in repeat copy number restricted to the unstable end of the tandem repeat array, and with evidence in some cases for interallelic transfer of repeats during mutation (data not shown). We therefore conclude that there is no obvious difference in mutation process between the control and exposed groups.

If enhanced mutation is the result of radiation exposure, it appears highly unlikely that minisatellites themselves are the

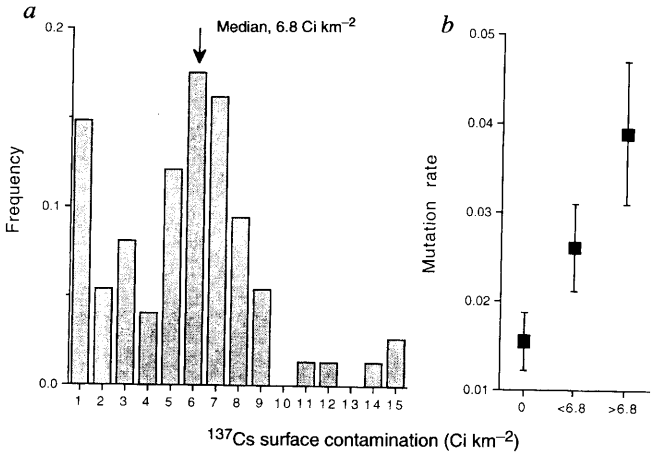

FIG. 4 ^{137}Cs surface contamination and mutation rate. *a*, Distribution of ^{137}Cs surface contamination in the place of residence of each Mogilev family analysed (data taken from ref. 30). *b*, Frequency of minisatellite mutation (±s.e.m., probes 33.15, MS32 and CEB1) in offspring grouped according to surface contamination (0, control population).

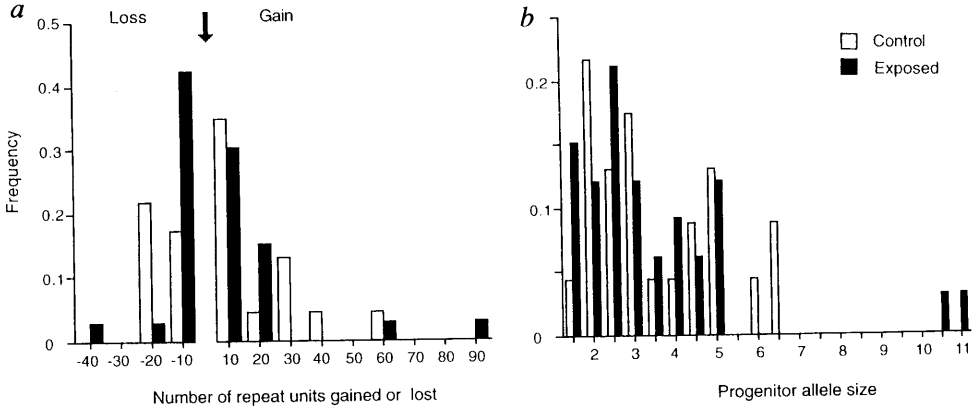

FIG. 5 Characteristics of minisatellite mutants detected by four single-locus probes. *a*, Distribution of fragment size changes (Kolmogorov–Smirnov test, $P > 0.05$). *b*, Distribution of progenitor allele sizes for mutants (Kolmogorov–Smirnov test, $P > 0.05$). The progenitor allele was assumed to be the parental allele closer in size to the mutant allele[9]; progenitors were verified for MS31 and MS32 mutants by MVR–PCR[17,18].

direct targets of irradiation[12]. The human haploid genome contains about 3×10^9 base pairs, and the average minisatellite scored by probe 33.15 has about 5,000 base pairs. The increase in mutation rate apparently caused by radiation was found to be about 0.01 per offspring band (probe 33.15; Table 1). If minisatellite mutations are initiated by double-strand breaks (DSBs)[18], then this increase would require 6,000 extra DSBs per haploid genome, assuming that minisatellite loci are random targets; no more than 70 DSBs are induced per cell per 1 Gy of irradiation[19]. This discrepancy is even greater for CEB1, which shows an increase in paternal mutation rate in the Mogilev group of 0.13 per sperm. It therefore seems that the increase in mutation rate is not caused by targeted events (DNA damage induced directly at minisatellites), but rather results form non-targeted effects caused by radiation elsewhere in the genome. One possibility is that radiation induces some system in the germline that in turn stimulates minisatellite instability, for example by interacting with a rate-limiting step in the complex gene conversion-like pathway known to be involved in mutation at human minisatellites[18]. It remains to be seen whether this system alters the mutation process itself, as seen for somatic mutations in two human protein-coding loci, which show a marked change in structural basis between spontaneous and radiation-induced mutation[20,21].

Discussion

We have used five minisatellite probes to estimate the mutation rate after the Chernobyl accident and found a doubling in mutation frequency among offspring of irradiated parents that may be a direct consequence of radiation exposure. Data collected in Hiroshima and Nagasaki during the past 40 years from the children of atomic bomb survivors, using eight different indicators, do not provide evidence of any statistically significant differences between exposed and control families[22,23]. We therefore believe that the present study provides the first experimental evidence that germline mutation rates in humans can be increased by ionizing radiation. These data have been obtained by a new approach using hypervariable loci with spontaneous mutation rates at least 1,000 times higher than in most protein-coding loci. Evidence for germline-mutation induction was obtained from a small sample (only 552 individuals from both the control and exposed groups), which is substantially lower than the sample size needed to detect the same increase in mutation rate by standard genetic techniques[24].

The dose–response curve for radiation-induced minisatellite mutation remains unknown. Estimates of radiation dose in rural populations of the Mogilev district suggest an average thyroid exposure by ^{131}I of about 0.185 Gy per person[2]. In contrast, the individual radiation dose for external and internal chronic exposure to ^{137}Cs was estimated to be less than 5 mSv per year[2], a value far below that predicted from mouse data[11–13,25] and current estimates of the doubling dose for humans[23]. It therefore seems either that the observed increase in minisatellite mutation rate, if resulting from radiation, was caused by the initial acute ^{131}I exposure, or that doses of chronic irradiation by ^{137}Cs have been substantially underestimated. Alternatively, it is possible that low doses of chronic irradiation are more effective in mutation induction than higher doses of acute irradiation[26,27]. Additional population surveys are needed to test whether ionizing radiation does induce minisatellite mutation, and to investigate the relative impact of acute and chronic radiation exposure on germline instability.

Note added in proof: A similar study of minisatellite mutation in a relatively small number of families of atomic-bomb survivors from Hiroshima and Nagasaki has failed to show evidence for mutation induction following acute exposure[31]. □

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Further evidence for elevated human minisatellite mutation rate in Belarus eight years after the Chernobyl accident.

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Abstract

Analysis of germline mutation rate at human minisatellites among children born in areas of the Mogilev district of Belarus heavily polluted after the Chernobyl accident has been extended, both by recruiting more families from the affected region and by using five additional minisatellite probes, including multi-locus probe 33.6 and four hypervariable single-locus probes. These additional data confirmed a twofold higher mutation rate in exposed families compared with non-irradiated families from the United Kingdom. An elevated rate was seen at all three independent sets of minisatellites (detected separately by multi-locus probes 33.15, 33.6 and six single-locus probes), indicating a generalised increase in minisatellite germline mutation rate in the Belarus families. Within the Belarus cohort, mutation rate was significantly greater in families with higher parental radiation dose estimated for chronic external and internal exposure to caesium-137, consistent with radiation induction of germline mutation. The spectra of mutation seen in the unexposed and exposed families were indistinguishable, suggesting that increased mutation observed over multiple lo arises indirectly by some mechanism that enhances spontaneous minisatellite mutation.

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Elevated Minisatellite Mutation Rate in the Post-Chernobyl Families from Ukraine

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Germline mutation at eight human minisatellite loci has been studied among families from rural areas of the Kiev and Zhitomir regions of Ukraine, which were heavily contaminated by radionuclides after the Chernobyl accident. The control and exposed groups were composed of families containing children conceived before and after the Chernobyl accident, respectively. The groups were matched by ethnicity, maternal age, parental occupation, and smoking habits, and they differed only slightly by paternal age. A statistically significant 1.6-fold increase in mutation rate was found in the germline of exposed fathers, whereas the maternal germline mutation rate in the exposed families was not elevated. These data, together with the results of our previous analysis of the exposed families from Belarus, suggest that the elevated minisatellite mutation rate can be attributed to post-Chernobyl radioactive exposure. The mechanisms of mutation induction at human minisatellite loci are discussed.

Introduction

Experimental evidence for radiation-induced germline mutation in humans still remains highly controversial. Because of a lack of experimental data on the effects of radiation exposure on germline mutation in humans, germline mutation induction in mice remains the main source of experimental data used to evaluate the genetic risk of human exposure to ionizing radiation (UNSCEAR 1993; Sankaranarayanan and Chakraborty 2000). Given that such an extrapolation currently cannot be verified, it remains clear that reliable estimates of the genetic risk of human exposure to ionizing radiation should be derived from relevant experimental data on germline mutation induction in human populations.

We have previously reported that minisatellite mutation rates in families inhabiting rural areas of the Mogilev region of Belarus, heavily contaminated with radionuclides after the Chernobyl accident, were unusually high (Dubrova et al. 1996, 1997). We also suggested that this increase could be attributed to the influence of environmental factors, including ionizing radiation. However, the results of this pilot study, in which mutation rates in the exposed group were compared with those in the nonexposed white families of different ethnic origins, do not provide enough evidence for induction of germline mutations by radiation. To verify the results of our previous study and to determine whether mutation rate in the germline of other post-Chernobyl cohorts is also elevated, we have extended this analysis to the group of exposed families inhabiting rural areas of the Kiev and Zhitomir regions of Ukraine.

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Subjects and Methods

Blood samples were collected in rural areas of Ukraine. Informed consent was obtained from all families included in this study. The detailed characteristic of families included in this study is given in the "Results" section.

DNA was purified from frozen blood using phenol-chloroform extraction. Four-microgram samples of DNA were digested to completion with *Alu*I, were electrophoresed through a 40-cm-long 0.8% agarose gel (SeaKem, type LE, FMC Products) in 1xTBE buffer (89 mM Tris-borate, pH 8.3, 2 mM EDTA), were transferred to a nylon membrane (Magna Nylon, Osmonics), and were hybridized to ^{32}P-labeled probes, as described elsewhere (Dubrova et al. 1996, 1997). All parents and offspring were profiled using eight hypervariable minisatellite probes CEB1, CEB15, CEB25, CEB36, MS1, MS31, MS32 (loci *D2S90, D1S172, D10S180, D10S473, D1S7, D7S21,* and *D1S8*), and B6.7 (located on chromosome 20q13), chosen for their high spontaneous mutation rate (Jeffreys et al. 1988; Tamaki et al. 1999; Vergnaud and Denoeud 2000). These probes were previously used for the analysis of human families from

Belarus who were exposed to the Chernobyl radioactive fallout (Dubrova et al. 1996, 1997), as well as families from Kazakhstan who were exposed to fallout from nuclear-weapon tests (Dubrova et al. 2002).

All autoradiographs were scored for the region between 1 and 22 kb, and new mutant bands were identified as offspring bands present in neither parent. DNA fragment sizes were estimated by the method of Southern (1979), using a 1-kb DNA ladder (Invitrogen) included on all gels. By use of the Chakraborty algorithm for parentage testing (Chakraborty et al. 1996), correct paternity for all offspring was validated. The likelihood ratio of paternity to nonpaternity in all families, including those with nonpaternal mutant bands ranged from 10^2 to 10^{11}.

Results

Population Groups

Blood samples were collected from 256 Ukrainian families inhabiting the rural areas of the Kiev and Zhitomir regions of Ukraine, which were radioactively contaminated following the Chernobyl accident (table 1; fig. 1). The control group was composed of 98 children conceived before the Chernobyl accident and born between 1976 and 1986. The exposed group contained 240 children conceived after the Chernobyl accident and born between 1987 and 1996. Fifty-four families included children conceived before the Chernobyl accident; 171 families included children conceived after the accident. An additional group of 27 families contained children conceived before and after the accident.

The control group contained 51 boys and 47 girls; the exposed groups consisted of 129 boys and 111 girls. The paternal age in the exposed group exceeded that for the control group (25.6 ± 0.4 and 26.8 ± 0.3 years for control and exposed groups, respectively; $t = 2.38$, $P = .0179$), which was mainly attributed to the families containing children conceived before and after the accident. The maternal ages in the control and exposed groups did not significantly differ (23.8 ± 0.4 years and 24.3 ± 0.3 for control and exposed group, respectively; $t = 0.97$, $P = .3324$). Table 2 gives the parental occupations for the three groups of families with children conceived before and after the Chernobyl accident, both paternal and maternal occupations were similar for all three groups. The number of smokers was also similar for these groups (table 2).

Figure 1 Map showing the study area

The data for the exposed group from Ukraine was also compared with our previous data on 125 families from the rural areas of the Mogilev region of Belarus who were affected by the Chernobyl fallout (Dubrova et al. 1996, 1997). The Belarus exposed group contained 61 boys and 64 girls born between February and September 1994 (for the sex ratio in the exposed groups from Ukraine and Belarus, $\chi^2 = 0.80$, 1 df, $P = .3703$). The paternal (27.6 ± 0.5 years) and maternal (24.5 ±

Table 1

Groups Studied

Location	No. of Families with Children Conceived			No. of Children Conceived	
	Before Chernobyl	After Chernobyl	Before and After Chernobyl	Before Chernobyl	After Chernobyl
Ovruch	15	94	21	42	146
Borodyanka	11	35	5	20	45
Gornostaipol	5	15	1	6	21
Vasylkov	23	0	0	30	0
Kovalevka	0	19	0	0	20
Ustimovka	0	8	0	0	8
Total	54	171	27	98	240

Table 2

Parental Occupation and Smoking Habits in Control and Exposed Families

Characteristic	No. (%) of Fathers from Families with Children Conceived			No. (%) of Mothers from Families with Children Conceived		
	Before Chernobyl	After Chernobyl	Before and After Chernobyl	Before Chernobyl	After Chernobyl	Before and After Chernobyl
Occupation (Code[a]):						
Management (1)	0	1 (.6)	0	1 (1.8)	11 (6.4)	1 (3.7)
Education and health (2)	5 (9.3)	14 (8.2)	2 (7.4)	24 (44.4)	77 (45.0)	11 (40.7)
Literary, artistic, and sport (3)	0	1 (.6)	0	0	1 (.6)	0
Science, engineering, technology (4)	2 (3.7)	7 (4.1)	2 (7.4)	1 (1.8)	7 (4.1)	4 (14.8)
Managerial (5)	5 (9.3)	11 (6.4)	1 (3.7)	1 (1.8)	1 (.6)	0
Clerical and related (6)	0	2 (1.2)	1 (3.7)	6 (11.1)	11 (6.4)	1 (3.7)
Selling (7)	1 (1.8)	0	0	7 (13.0)	6 (3.5)	1 (3.7)
Security and protective service (8)	3 (5.6)	9 (5.3)	0	0	1 (.6)	1 (3.7)
Catering, cleaning, and hairdressing (9)	0	2 (1.2)	0	5 (9.3)	14 (8.2)	4 (14.8)
Farming (10)	3 (5.6)	13 (7.6)	0	0	7 (4.1)	0
Material processing (11,12)	12 (22.2)	34 (19.9)	6 (22.2)	5 (9.3)	11 (6.4)	1 (3.7)
Painting, assembling, and packaging (13)	1 (1.8)	0	1 (3.7)	1 (1.8)	2 (1.2)	0
Construction (14)	4 (7.4)	13 (7.6)	2 (7.4)	1 (1.8)	3 (1.8)	1 (3.7)
Transport (15)	12 (22.2)	52 (30.4)	12 (44.4)	0	3 (1.8)	0
Miscellaneous (16)	1 (1.8)	3 (1.8)	0	1 (1.8)	6 (3.5)	0
Inadequately described (17)	5 (9.3)	9 (5.3)	0	0	1 (.6)	0
Housewives	...	...	...	1 (1.8)	9 (5.3)	2 (7.4)
Smoking Status:						
Non-smokers	24 (44.4)	53 (31.0)	7 (25.9)	53	166	27
Smokers	30 (55.6)	118 (69.0)	20 (74.1)	1	5	0

Note.—For data on fathers' occupations, $\chi^2 = 6.78$; 6 df; $P = .3421$; for data on mothers' occupations, $\chi^2 = 5.52$; 6 df; $P = .4795$; and for data on fathers' smoking status, $\chi^2 = 5.52$; 2 df; $P = .1404$.

[a] Occupational codes are taken from Office of Population Censuses and Surveys (1980).

0.5 years) ages in the exposed group from Belarus did not significantly differ from those in the exposed group from Ukraine (for paternal age, $t = 1.31$ and $P = .1914$; for maternal age, $t = 0.28$ and $P = .7770$). The parental occupations in the exposed groups from Ukraine and Belarus were also similar (for paternal occupation, $\chi^2 = 6.77$, 6 df, $P = .3426$; for maternal occupation, $\chi^2 = 9.31$, 5 df, $P = .0972$).

Mutation Rate

A summary of all mutation data is presented in table 3. A statistically significant 1.6-fold increase in the paternal mutation rate was found in the exposed families from Ukraine, whereas maternal mutation rate in this cohort was not elevated. Most of the minisatellite loci showed an elevated paternal mutation rate in the exposed group (fig. 2*A*).

Using the same experimental procedures, we have previously analyzed minisatellite germline mutation rates in the exposed families from Belarus and nonirradiated white families from the United Kingdom (Dubrova et al. 1996, 1997). Figure 2*B* presents the comparison of our current and previous results, obtained using the same eight minisatellite probes. Despite differences in ethnicity, the nonexposed groups of parents from Ukraine and the United Kingdom showed very similar minisatellite mutation rates in the paternal ($P = .8662$, Fisher's exact test) and maternal ($P = .7903$) germlines. Most importantly, the estimates of paternal and maternal mutation rates in the exposed groups from Ukraine and Belarus were indistinguishable ($P = .5204$ and $P = .4938$ for paternal and maternal mutation rates, respectively). We therefore conclude that minisatellite mutation rates in the germlines of exposed fathers from Ukraine and Belarus are significantly elevated, whereas maternal mutation rates in these two exposed cohorts do not differ significantly from those in the nonexposed mothers.

Paternal Age, Year of Conception, and Paternal Mutation Rate

The paternal age in the exposed groups from Ukraine and Belarus exceeded that for the control group from Ukraine. This difference could potentially explain an elevated mutation rate in the exposed cohort from Ukraine. The results of numerous studies show the age-related increase in paternal germline mutation rate at human protein-coding genes (Vogel and Rathenberg 1975), although there has been no data showing such a trend for human minisatellites. To determine whether the increased mutation rate in the exposed fathers might

Table 3

Mutation Rates in Control and Exposed Groups

	Findings in Control Group			Findings in Exposed Group				
Probe	No. of Mutations	No. of Bands	Rate	No. of Mutations	No. of Bands	Rate	Ratio[a]	p[b]
Paternal mutations:								
B6.7	3	82	.0366	18	196	.0918	2.51	...
CEB1	16	90	.1778	38	219	.1735	.98	...
CEB15	2	87	.0230	18	218	.0826	3.59	...
CEB25	3	93	.0323	10	224	.0446	1.38	...
CEB36	1	65	.0154	5	191	.0262	1.70	...
MS1	2	94	.0213	13	223	.0583	2.74	...
MS31	2	98	.0204	4	239	.0167	.82	...
MS32	0	97	0	6	238	.0252	...	...
Total	29	706	.0411	112	1748	.0641	1.56	.0299
Maternal mutations:								
B6.7	0	80	0	4	196	.0204	...	...
CEB1	1	86	.0116	2	208	.0096	.83	...
CEB15	0	91	0	3	211	.0142	...	...
CEB25	1	91	.0110	2	229	.0087	.79	...
CEB36	1	73	.0137	2	179	.0112	.82	...
MS1	7	88	.0795	12	226	.0531	.67	...
MS31	0	98	0	0	240	0	...	...
MS32	0	94	0	0	238	0	...	...
Total	10	701	.0143	25	1727	.0145	1.01	1

[a] Exposed:control ratio.

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

be age-related, a correlation analysis for the control and exposed groups from Ukraine and Belarus was performed, which failed to reveal any significant associations between the paternal age and mutation rate (fig. 3*A*). Importantly, an elevated mutation rate was found across most of the age cohorts of exposed fathers. We therefore conclude that the relatively small difference in the paternal age between the exposed and control groups cannot explain the increased mutation rate in the exposed fathers.

In our previous study, the frequency of minisatellite mutation was evaluated in a cohort of Belarus children born within a narrow interval (February–September 1994), which prevented the analysis of temporal changes in mutation rate following the Chernobyl accident (Dubrova et al. 1996, 1997). The current cohort of children from Ukraine were conceived between 1986 and 1995, which makes such analysis possible. Figure 3*B* represents the scatter plot of paternal mutation rate. In the control and exposed groups, paternal mutation rates were uniform across the paternal cohorts (for homogeneity of the Poisson distribution, $\chi^2 = 1.11$, 3 df, $P = .7747$ for control fathers and $\chi^2 = 2.16$, 7 df, $P = .9504$ for exposed fathers) and did not show any correlation with the year of conception. We can therefore conclude that, within the analyzed period of time, paternal mutation rate in the control and exposed groups from Ukraine remained stable before and after the Chernobyl accident, respectively.

Spectrum of Paternal Minisatellite Mutation

Figure 4*A* presents the distribution of paternal progenitor alleles in the control and exposed groups from Ukraine and Belarus. The progenitor allele was assumed to be the paternal allele closest in size to the mutant allele (Jeffreys et al. 1988). The sizes of progenitor alleles were similar in the three groups, which allowed further comparison of mutation spectrum (fig. 4*B*). The results obtained here are in good agreement with the results of previous studies analyzing spontaneous mutation at these minisatellite loci (Jeffreys et al. 1988, 1994; Buard et al. 1998; Tamaki et al. 1999; Vergnaud and Denoeud 2000), most mutation events involved the gain or loss of only few repeat units. The distributions of length changes were indistinguishable between the three groups. We therefore conclude that there is no obvious difference in the spectrum of paternal minisatellite mutations between these groups.

Discussion

The main results of our study show that the paternal mutation rate at eight minisatellite loci in the exposed families from Ukraine is elevated, and they do not provide evidence for elevated mutation rates in the germline of exposed mothers. In this section, we will compare the Ukrainian data with the results of our previous study of the Belarus families inhabiting the rural areas of the

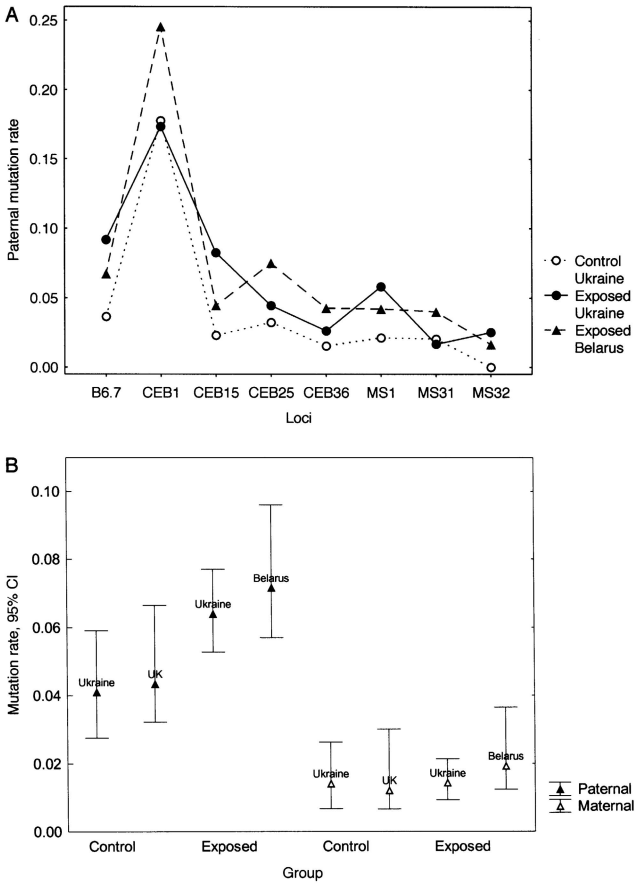

Figure 2 Minisatellite germline mutation rates in the control and exposed groups from Ukraine, Belarus, and the United Kingdom. *A,* Single-locus estimates of paternal mutation rates in the control and exposed groups from Ukraine (Wilcoxon matched pairs test, $Z = 2.10$; $P = .0357$) and exposed group form Belarus ($Z = 2.52$; $P = .0117$). *B,* Comparison of paternal and maternal minisatellite germline mutation rates in the exposed and nonexposed families of different origin. Data for the Belarus and U.K. families are taken from previous studies by Dubrova et al. (1996, 1997).

Mogilev region, which was also contaminated after the Chernobyl accident (Dubrova et al. 1996, 1997), and we will address some issues concerning mutation induction at human minisatellite loci.

For this study, the control and exposed groups from Ukraine were matched by ethnicity, maternal age, parental occupation, and smoking habits, and they differed only slightly with respect to paternal age. Analysis of this potentially confounding factor shows that it does not affect minisatellite mutation rates in the germlines of control and exposed fathers (fig. 3*A*). Such a design provides a much better control population than was used for the previous study of the Belarus families, in which mutation rates in the exposed group were compared with those in the nonexposed white families of different ethnic origins. Importantly, the Ukrainian families do not significantly differ by parental age and occupation from the Belarus families inhabiting geographically similar areas of the Mogilev region, which was also contaminated after the Chernobyl accident.

A statistically significant 1.6-fold increase in mutation rate was found in the germline of exposed fathers from Ukraine. For this set of loci, paternal mutation rate in the exposed families from Belarus was 1.7 times higher than in the control group from Ukraine ($P = .0121$). In both exposed groups, the mutation rate was elevated over a number of minisatellite loci. Since the control group from Ukraine and both the exposed groups from Ukraine and Belarus were matched for parental age and occupation, the elevated mutation rate found in the ex-

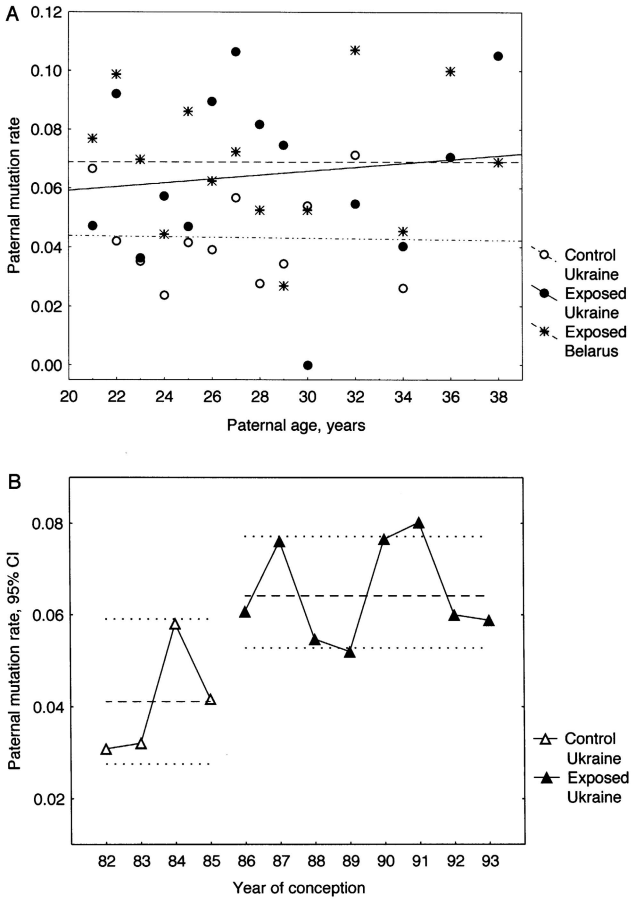

Figure 3 Minisatellite paternal mutation rate, paternal age and the year of conception in the control and exposed groups from Ukraine and Belarus. *A,* The lack of correlation between mutation rate and the paternal age in the control and exposed fathers (Kendall's nonparametric correlation: control Ukrainian fathers, $\tau = -0.091$, $P = .6808$; exposed Ukrainian fathers, $\tau = 0.033$, $P = .8695$; exposed Belarus fathers, $\tau = -0.110$, $P = .5820$). *B,* Paternal mutation rate and the year of conception in the control and exposed group from Ukraine. The dashed lines represent paternal mutation rates (± 95% CI) for the whole control and exposed groups. Data for the Belarus families are taken from previous studies by Dubrova et al. (1996, 1997).

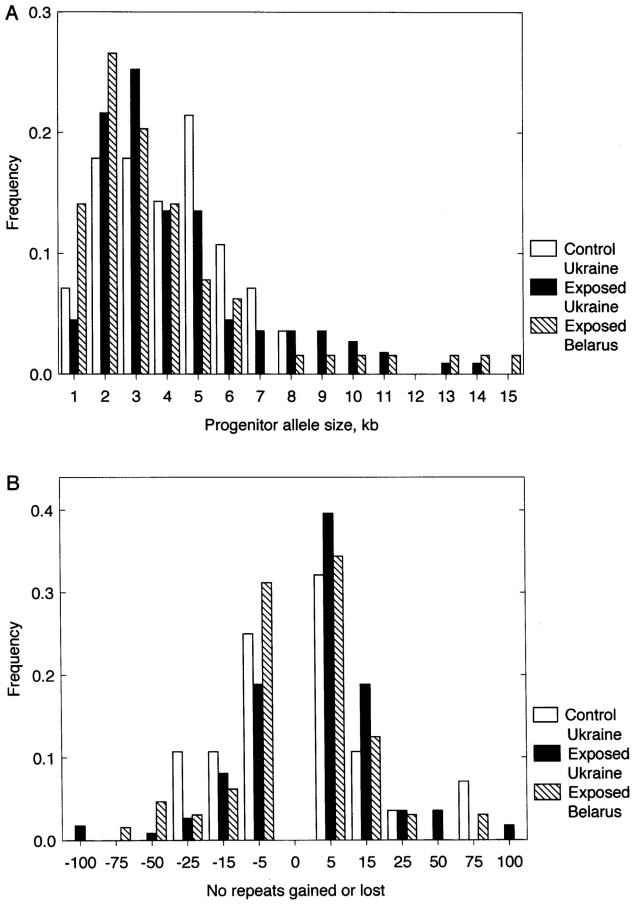

Figure 4 Spectrum of paternal germline mutations in the control and exposed groups from Ukraine and Belarus. *A,* Distribution of progenitor allele sizes for mutants (Kolmogorov-Smirnov test for all paired comparisons, $P > .10$; Kruskal-Wallis ANOVA, $H = 3.09$; $P = .2129$). *B,* Distribution of size changes for paternal mutations (Kolmogorov-Smirnov test for all paired comparisons, $P > .10$; Kruskal-Wallis ANOVA, $H = 3.27$; $P = .1953$). Data for the Belarus families are taken from previous studies by Dubrova et al. (1996, 1997).

posed families could be attributed to the influence of environmental mutagens.

Over 90% of children from the exposed group were born in Ovruch, Borodyanka, and Gornostaipol, which belong to the most heavily radioactively contaminated areas of Ukraine with a level of surface contamination from cesium-137 of >2 Ci/km^2 (IAC 1991). According to gamma spectrometric measurements of radionuclide concentration in soil and measurements of external gamma-exposure rate in air, the whole-body doses from external sources for the rural population of contaminated areas of Ukraine for the period of time from 1986 to 2000 did not exceed 50 mSv (Likhtarev et al. 2002). Similar doses from the ingestion of cesium-137 and cesium-134 for the Ukrainian population were also reported (Likhtarev et al. 2000). These doses are well below all known estimates of the doubling dose for mammalian germline mutation of 1 Sv (UNSCEAR 1993; Sankaranarayanan and Chakraborty 2000) and therefore cannot explain the 1.6-fold increase in mutation rate found in the exposed families. It should be noted, however, that the estimated doses reflect only one component of human exposure and do not take into account internal exposure from the short-lived isotopes.

The Chernobyl accident resulted in an unprecedented release of a wide spectrum of short-lived isotopes with the level of deposition often an order of magnitude higher than that for cesium-137. During the first days after the Chernobyl accident, their contribution to the absorbed dose in air was extremely high (Golikov et al. 1993), and the initial dose rate in air within the 100-km zone was at least 20-fold higher than in the later years (Balonov 1993). The high doses of internal and external exposure from the short-lived radionuclides were also reported for the residents of the 30-km zone around the Chernobyl power plant evacuated within 3–11 d after the accident (Pröhl et al. 2002). It should be noted that, apart from iodine-131, the contribution of short-lived radionuclides to the total exposure of nonevacuated population of Ukraine and Belarus has never been analyzed.

Given the short half-life of unstable radionuclides, their contribution to external and internal exposure can no longer be evaluated by means of physical dosimetry. However, retrospective biological dosimetry provides an alternative approach for the evaluation of the total absorbed doses for the populations of contaminated territories. The analysis of stable and unstable chromosome aberrations has provided an estimate of the mean doses of exposure, for the residents of heavily contaminated areas of the Gomel region of Belarus, ranging between 0.2 and 0.4 Grays (Gy) (Darroudi and Natarajan 1996; Mikhalevich et al. 2000). The reconstructed doses for the evacuees from the 30-km exclusion zone, received within a few days after the Chernobyl accident, were 0.3 and 0.4 Gy for the cohorts from Ukraine and Belarus, respectively (Maznik et al. 1997; Mikhalevich et al. 2000). These estimates are markedly higher than those obtained by physical dosimetry and probably reflect the initial external and internal exposure to the short-lived radionuclides. If we assume that the doubling dose for germline mutation in humans is 1 Gy (UNSCEAR 1993; Sankaranarayanan and Chakraborty 2000), an exposure to 0.2-0.4 Gy could potentially lead to a 1.6-fold increase in minisatellite mutation rate as found in the families from Ukraine and Belarus. Our data also suggest that the elevated paternal mutation rate found in the Ukrainian cohort of exposed families may be attributed to the initial exposure. Thus, within this cohort, the paternal mutation rate remains stable over the period of time from 1986 to 1994 and exceeds

that for the control group. If the chronic exposure from cesium-137 and other stable radionuclides caused the elevated mutation rate, then a positive correlation between the year of conception and mutation rate should occur in the Ukrainian cohort. On the contrary, the consistently elevated paternal mutation apparently reflects a relatively high initial exposure for this cohort with a small contribution from the stable nuclides following the decay of the short-lived isotopes.

Our data therefore show that the elevated mutation rate in the germline of exposed fathers is most likely radiation induced and raise the issue of mechanisms of mutation induction at minisatellite loci. We have hypothesized that mutation induction at human minisatellites cannot be attributed to the direct effects of radiation-induced DNA double strand breaks at these small genomic loci (Dubrova et al. 1996, 1997), also similar conclusions have been obtained from studies of somatic and germline mutation rates at other DNA repeat sequences (Sadamoto et al. 1994; Schiestl et al. 1994; Fan et al. 1995; Dubrova et al. 1998, 2000; Barber et al. 2000; Kovalchuk et al. 2000; Yauk et al. 2002). The main argument for nontargeted mechanisms reflects the fact that an unrealistically high number of extra double-strand breaks or other types of damage per genome would be required to explain the mutation induction observed at these loci (details of estimates are given by Schiestl et al. [1994] and Dubrova et al. [1998]). Other evidence for the nontargeted mechanisms comes from the comparison of the spectrum of minisatellite mutation in the germline of control and exposed parents. In contrast to protein-coding genes showing a marked change in the structural basis of spontaneous and radiation-induced mutation (Nelson et al. 1994; Giver at al. 1995), human minisatellite loci and mouse expanded simple tandem repeat loci display indistinguishable mutation spectra in the germline of nonexposed and exposed parents (Dubrova et al. 1996, 1997; Yauk et al. 2002). Our current data also show similar mutation spectra in the control and exposed families, suggesting that there is no obvious difference in mutation process between the two groups.

Current data on the processes of spontaneous mutation at the human GC-rich minisatellites may provide clues to the mechanisms of mutation induction at these loci. Spontaneous mutation at these loci is very complex and almost completely restricted to the germline, with very rare and simple mutational events occurring in the somatic cells (Jeffreys at al. 1994; May et al. 1996; Jeffreys and Neumann 1997; Tamaki et al. 1999; Buard et al. 2000; Stead and Jeffreys 2000). These data also show that minisatellite mutation in the paternal germline most likely occurs at meiosis. If so, then exposure to ionizing radiation could potentially affect the stability of minisatellite loci over a very short interval of meiosis. To judge from the results of our study, this possibility appears to be highly unlikely. Given a relatively high initial exposure from the short-lived radionuclides, the preferential targeting of meiosis should result in a considerably elevated paternal mutation rate during the 1st year after the Chernobyl accident. On the contrary, the mutation rate in the exposed fathers from Ukraine remains stable over the period of time from 1986 to 1994. It therefore appears that premeiotic diploid stem cells and spermatogonia could accumulate radiation-induced damage, elsewhere in the genome, that subsequently affects the stability of minisatellite loci at meiosis. This delayed stimulation of minisatellite mutation in meiotic cells is reminiscent of the phenomenon of radiation-induced genomic instability, in which ionizing radiation can not only induce mutations seen in directly exposed somatic cells but can also lead to delayed effects with new mutations arising many cell divisions after the initial irradiation damage (Morgan et al. 1996).

In contrast to the increased mutation rate in exposed fathers, mutation rate in the germline of exposed mothers from Ukraine and Belarus is not elevated. This may be attributed to the relatively low maternal mutation rates at the most of the minisatellite loci, which do not provide enough statistical power to detect an elevated mutation rate in the exposed cohorts. Indeed, given the wide CI of the ratio of maternal mutation rates in the exposed group to control (95% CI 0.38–2.00), we cannot exclude the possibility of an elevated mutation rate among the irradiated mothers. However, the profound differences in the timing of spermatogenesis and oogenesis may explain the apparent similarity in mutation rates in the germline of exposed and nonexposed mothers. Spermatogenesis is a continuous process of mitotic and meiotic cell divisions, occurring from the beginning of puberty, whereas oocytes are already formed in late embryogenesis and remain arrested until the onset of puberty (Vogel and Motulsky 1997). Most importantly, crossing-over in the maternal germline also occurs in the late embryonic stages. If the mechanisms of minisatellite mutation in females are similar to those in males, then minisatellite mutation in the maternal germline may only occur before birth. All the mothers included in our study were at least 8 years old at the time of the Chernobyl accident, implying that they had been irradiated during the late meiotic stages, where exposure to ionizing radiation may be unable to affect the stability of minisatellite loci in the maternal germline.

In conclusion, the results of our current and previous studies show that mutation rate in the germline of fathers from Ukraine and Belarus is indeed elevated, and these results provide strong evidence that this increase can be attributed to post-Chernobyl radioactive exposure. The main questions remaining concern doses for the population of contaminated territories and the

mechanisms of radiation-induced mutation at human minisatellites. Future work should address these important issues.

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