

Report of the COMARE subgroup on Molecular Endpoints of Radiation Dose/Effect

1. Summary

1.1 This report considers recent data, largely from mouse and human studies, on mutational risk to the germline as detected by molecular analysis of tandemly repeated DNA loci (TRDLs, specifically Expanded Simple Tandem Repeats (ESTRs) in mice and minisatellites in humans). Mutations at these loci are generally detected in the offspring of control and irradiated parents and can also be directly detected in the paternal germline (sperm). Little is known of the mechanisms by which mouse ESTR and human minisatellite mutations occur - despite superficial similarity the processes in the two systems may be distinct. Consequently caution should be taken in using mouse ESTR data to predict human minisatellite effects. Specific variants of minisatellites have been found to associate with some human diseases and have effects on the expression of genes. Where repeat sequence mutations alter gene structure or expression leading to altered disease predisposition then these effects are covered by current models for genetic risk following radiation exposure. However, what other effects on human health might be caused by minisatellite variants, including those induced by radiation, is unclear at present.

1.2 Specifically:

- TRDL mutation has been used as a marker of human radiation exposure in areas highly contaminated with internal emitters, but it has proven negative with radiation sources that are predominately external.
- Studies using mice suggest that TRDL mutation might be more widely applicable for biomonitoring but there are important differences between the human and mouse systems.
- Some minisatellite variants are associated with human disease and may affect critical processes. However, caution is required because little direct evidence links minisatellite variation to disease and no specific health effects have been demonstrated as a consequence of radiation-induced minisatellite mutations. A generalisation of potential health effects of TRDL mutation should not be made at the present stage of our understanding.
- Despite our lack of knowledge regarding the implications of TRDL mutations, the epidemiological data indicate that it is highly improbable that a dramatic revision of risk estimates for cancer will be needed in the light of TRDL mutation studies. However, these mutations may play a role in rare conditions and other processes such as pregnancy, development or viral disease; only further research will determine this.
- The precise mechanisms whereby TRDL mutations arise remain obscure.
- In mice and humans the mutations are untargeted with no clear dose response although two papers have suggested a linear dose-response in mice.
- Evidence for transgenerational mutation has been obtained for mice but not for humans.

2. Context

2.1 In its seventh report COMARE (2002) considered cancer risk in the children of parents occupationally exposed to ionising radiation prior to conception. This report extended the earlier fourth COMARE (1996) report which specifically considered the Gardner Report (Gardner, 1990) and available data relevant to the hypothesis that paternal preconceptional irradiation is associated with an increased risk of childhood leukaemia in subsequent offspring. Included in the fourth and seventh reports is the recognition of recent studies indicating that non-targeted mutational effects of radiation may occur in the male mouse germline. Effects of this type could in principle contribute to cancer risk in the offspring of irradiated parents. Despite finding little evidence for a parental pre-conceptional

radiation (PPI) effect in mouse and human studies in general, recommendations for further study of germline mutation were made. Two specific recommendations in the seventh report are relevant here:

Recommendation 1

“Some radiation-induced changes in the genome in repeat DNA sequences have been observed experimentally to occur in germ cells of male mice at frequencies higher than hitherto suspected, implying that some regions of the genome may be more susceptible to ionising radiation. We recommend that experiments be undertaken to elucidate the underlying mechanisms behind these observations. This will aid in establishing what, if any, effect such changes may have on current estimates of risk associated with radiation exposure.”

Recommendation 2

“Currently, there is little understanding of how mutations in certain repeat DNA sequences might affect human health. We recommend that basic research in this area should be encouraged.”

2.2 On the strength of these recommendations, COMARE formed a subgroup on Molecular Endpoints of Radiation Dose/Effect (MERDE) with the following remit:

“Genetic risk factors for ionising radiation are based on phenotypic and cytogenetic data in the mouse and are related to spontaneous mutation rates in humans. There are no useful data on radiation-induced mutation rates in humans. Within the last ten years molecular methods for detecting DNA sequence changes in sperm or offspring of irradiated parents have come into use. It is proposed to set up a COMARE subgroup with a remit to (i) examine the validity of the use of these endpoints for monitoring exposure of humans or the mutagenic potential of a particular environment, (ii) to assess the implications of these endpoints for human health.”

2.3 This report summarises the information currently available from molecular genetic and related studies on male germline mutation. Mechanisms of mutation are considered as are various aspects linking mutation to human health effects.

3. Introduction

3.1 Mutations are alterations in the sequence of DNA, recognised as heritable changes in cells and organisms. It is generally accepted that most mutations arise from DNA damage that leads to mistakes or causes slippage during DNA replication, or triggers recombination resulting in loss, gain or realignment of DNA sequences (Friedberg et al, 1995). DNA damage occurs continually due to cellular metabolism, for example through the production of active oxygen species, or alternatively as a result of exposure to exogenous agents such as radiations or chemicals.

3.2 There is a lack of positive evidence indicating an increase in human germline mutation following exposure to radiation from the atomic bombings in Japan (Neel et al, 1990). Consequently, current genetic risk estimates for radiation are based on measurement of mutation frequencies in the mouse using a handful of “marker genes” (Neel and Lewis, 1990; Sankaranarayanan, 2001). For example, mutations in genes controlling mouse fur colour can be recognised easily and show a dose-dependent increase following irradiation. However, the naturally-occurring frequency of such gene mutations is about 1 in 100,000 mice, necessitating very large experiments to determine frequency increases. Based on this relatively low frequency of mutations and extensive data from somatic mammalian cells, it was considered that ionising radiation-induced mutations arose at, or close to, the sites of damage.

3.3 In recent years, analysis of mouse and human DNA sequence has shown that some regions containing mainly repeat sequences collectively known as tandemly repeated DNA loci (TRDLs) mutate at a much higher frequency (x 1000) than the well-known marker genes (Bois and Jeffreys, 1999). Although the functions of these highly-mutable sequences are not well understood, and may differ in the mouse and human, they can act as markers of mutation induction. Additionally, the very high frequencies of radiation-induced mutation observed in mouse suggest that these mutations are not linked directly to sites of DNA damage; ie, the mutations are “untargeted”. Other types of experiment show that radiation may also cause changes in cells, including mutations, long after the

radiation exposure has finished (see, for example Little, 2003). 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.

3.4 Analysis of TRDLs provides an important new method for following mutations in the mouse and human germline (Yauk 2004). In humans the highly-mutable sequences occur at certain “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.

4. Biology of tandemly repeated DNA loci

4.1 Tandemly repeated DNA loci in the mammalian genome are represented by relatively short microsatellites (<500 bp) with repeat size of 1-4 bp, long expanded simple tandem repeats (ESTRs, 0.5-16 kb, repeat size 4-9 bp) and true minisatellites (0.5-10 kb) with repeat size of 9-60 bp (reviewed in: Ellegren, 2004; Bois and Jeffreys, 1999; Vergnaud and Denoeud, 2000). 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. In this section results from a number of publications on the biology of minisatellites and ESTR loci are summarised.

Minisatellite loci

4.2 Minisatellites consist of 9-60 bp repeats which show considerable sequence variation along the array (Jeffreys et al, 1991, 1994; Buard et al, 1998; Tamaki et al, 1999; Vergnaud and Denoeud, 2000). 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 (Jeffreys et al, 1999; Amarger et al, 1998). In addition, germline mutation rates at human minisatellite loci are substantially higher than those in the pig, rat and mouse genomes (Vergnaud et al, 1991).

4.3 To date, minisatellite mutation rates in humans have been evaluated using either the pedigree approach (Jeffreys et al, 1988) or small-pool PCR technique (Jeffreys et al, 1994; Buard et al, 1998; Tamaki et al, 1999). The latter 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 (Jeffreys et al, 1994). It has mainly been used for the analysis of mechanisms of minisatellite mutation in the paternal germline (Jeffreys et al, 1994; Buard et al, 1998; Tamaki et al, 1999). The pedigree-based analysis of minisatellite loci remains the only source of experimental data on minisatellite mutation in the maternal germline.

4.4 Mutation rates at minisatellites are locus-specific and show considerable variation between the paternal and maternal germ lines (Table 1). For the majority of human minisatellite loci, an excess of paternal mutations has been found. On average, the minisatellite mutation rate in the paternal germline is ~4 times higher than in the maternal germline. A similar sex bias has also been reported for human microsatellite loci (Ellegren, 2004). 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).

Table 1. Spontaneous mutation rates at the most unstable human minisatellite loci

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	<i>D2S90</i>	39	0.127 (0.101-0.144)	0.004 (0-0.012)	36.5
CEB15	<i>D1S172</i>	18	0.031 (0.014-0.056)	0.004 (0-0.012)	8.7
CEB25	<i>D10S180</i>	52	0.038 (0.027-0.065)	0.007 (0-0.012)	5.3
CEB36	<i>D10S473</i>	42	0.008 (0-0.015)	0.014 (0-0.025)	0.5
MS1	<i>D1S7</i>	9	0.042 (0.021-0.090)	0.049 (0.034-0.080)	0.8
MS31	<i>D7S21</i>	20	0.012	0	-
MS32	<i>D1S8</i>	29	0.010	0	-
Mean	-	-	0.043	0.012	3.8

Data from: Dubrova et al, 1997, 2002a, 2002b; Livshits et al, 2001; Kiuru et al, 2003; Kodaira et al, 2004.

4.5 Analyses of minisatellite loci have shown that the mutational processes in somatic tissue and the germline are different. High frequency mutation of these loci is almost completely restricted to the germline, with very rare and simple mutational events occurring in the somatic cells (Jeffreys et al, 1994; Jeffreys and Neumann, 1997; Tamaki et al, 1999; Buard et al, 2000a). Germline mutation at human minisatellites is attributed to complex gene-conversion-like events, often resulting in multiple mutational changes. No single model has yet been developed to account for all of 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 to give intra-allelic exchange (Jeffreys et al, 1999). It has also been shown that the mutation process at some minisatellite loci may be driven by an adjacent hotspot of meiotic recombination (Jeffreys et al, 1998a, 1998b; Buard et al, 2000b).

4.6 Some minisatellites, possibly up to 30-40% of the total (Denoëud et al, 2003), 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 (Mahley et al, 1984) and human epithelial mucin (Lancaster et al, 1990). The presence of minisatellites in the vicinity of the H-ras gene could affect its transcription and may predispose to heritable forms of cancer (Phelan et al, 1996). Minisatellites within intronic regions of genes have been shown to interfere with exon splicing; this may be due to the similarities of the consensus repeat of the minisatellite to splice donor repeat sequences, as is seen for the human interferon-inducible gene 6-16 (Turri et al, 1995). Although at present 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 functional role.

Expanded simple tandem repeat loci

4.7 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 (Bois et al, 1998a,b). Unstable ESTRs consist of homogenous arrays of relatively short repeats (4-9 bp) and, in contrast to minisatellites, show a very high spontaneous mutation rate both in germline and somatic cells (Kelly et al, 1989; Gibbs et al, 1993; Bois et al, 1998b). Currently ESTRs have only been identified in the mouse genome, but are thought to be present in other genomes including humans. It has been shown that ESTR loci are derived from highly expanded interspersed repeat elements in the mouse genome (Bois et al, 1998b). In contrast to human minisatellites, ESTR loci do not show any preferential telomeric clustering and are more or less randomly distributed in the mouse genome (Bois et al, 1998b).

4.8 To date, ESTR mutation rates in the mouse germline have been evaluated using the pedigree approach (reviewed in: Dubrova and Plumb, 2002; Dubrova, 2005). 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 (Yauk et al, 2002).

4.9 In contrast to human microsatellites and minisatellites, spontaneous ESTR germline mutation rates in mice do not show extensive paternal bias (Table 2). The high somatic mutation rate at ESTR

loci leads to high levels of mosaicism, eg, between 2.8% and 20% of adult mice possess more than two alleles at the mouse ESTR loci *Ms6-hm* and *Hm-2* (Kelly et al, 1989; Gibbs et al, 1993). 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 (Kelly et al, 1989; Gibbs et al, 1993).

Table 2. Spontaneous mutation rates at the most unstable mouse ESTR loci

Strain	<i>Ms6-hm</i> (GGCAG) _n			<i>Hm-2</i> (GGCA) _n			<i>Ms6-hm + Hm-2</i>		
	Paternal	Maternal	Ratio*	Paternal	Maternal	Ratio*	Paternal	Maternal	Ratio*
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 [†]	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

* Ratio of paternal mutation rate to maternal.

[†] C57BL/6 x CBA F₁

Data from: Dubrova et al, 2000a; Barber et al, 2000, 2002; Vilarino-Guell et al, 2003.

4.10 It has been proposed that spontaneous ESTR mutation is most probably attributable to replication slippage, similar mechanisms are suggested for microsatellite instability (Yauk et al, 2002; Barber et al, 2004; Dubrova, 2005). According to this model, the very high spontaneous mutation rate at some ESTR loci could be directly related to their very large size (500-3500 repeats) and, probably, the presence of hairpin structures within the arrays. These together may cause replication pausing and, subsequently, promote DNA polymerase slippage. This model is consistent with the observed positive correlation between spontaneous germline mutation rates and the sizes of ESTR loci (Bois et al, 2001).

4.11 To summarise, 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 behaviour of minisatellite loci dramatically differs 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.

Table 3. Properties of tandem repeat DNA loci

	Minisatellites	ESTRs	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	- ?	- random
Somatic instability	Very low	High	High
Mutation mechanism			
- germline	Meiotic recombination	Replication slippage?	Replication slippage
- somatic cells	?	Replication slippage?	Replication slippage

What implications do the mouse ESTR data have for mutation in humans?

4.12 It is critical to determine the relationship between the ESTR mutations in mice and minisatellite mutation data in humans. As summarised above, there are important differences in structure between mouse ESTRs and human minisatellites. Clearly, further study is required to understand the differences between different types of TRDL, particularly to define further the molecular mechanisms by which the mutations arise. However, beyond mechanistic questions, it is necessary to establish the following:

- i) the scope, strength and significance of the human and mouse studies of radiation-induced TRDL mutation
- ii) the relationship between the mouse and the human data
- iii) the potential impact of these mutations on human health.

These topics are the subject of this report.

4.13 To facilitate discussion of these issues, we have subdivided our report into the following categories:

Human studies of minisatellite mutation.
Radiation-induction of ESTR mutation in germ cells of male mice.
Transgenerational mutation studies.
Environmental monitoring studies.
Mechanisms of tandem repeat DNA locus mutation.
Potential implications of minisatellite mutation for human health.

4.14 Very important in the current context is transgenerational mutation which is restricted to situations where it can be unambiguously determined that the mutational event has occurred in the cells of the offspring rather than in the parental germ line. Therefore studies of germline mutations detected in the first generation offspring of irradiated parents are not termed transgenerational here because it is possible, and in some cases clear, that the mutational event occurred during germ cell maturation.

5. Human studies of minisatellite mutation

5.1 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 pre-meiotic diploid germ cells which 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 following 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.

5.2 To date, using the pedigree approach, germline minisatellite mutation rates have been evaluated in four irradiated groups from Japan and the former USSR. 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 hybridisation 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 Hiroshima and Nagasaki atomic bomb survivors

5.3 Minisatellite mutation rates have been evaluated in the exposed and control families from Hiroshima and Nagasaki (Kodaira et al, 1995; 2004). Key features of these studies:

- A relatively small group of 62 children (30 of exposed fathers and 32 of exposed mothers) and 60 children of non-exposed parents were analysed;
- The doses of parental exposure were reconstructed using Dosimetry System 86 (DS86);
- The exposed group with mean parental dose of 1.9 Sv was composed mostly of families with only one irradiated parent;
- Most of the children from the exposed families were born >10 years after the bombings;
- In the first study, four of six probes used detected loci with a very low mutation rate (Kodaira et al, 1995);

- In the second study, germline mutations were scored using eight single-locus probes detecting the most unstable human minisatellite loci (Kodaira et al, 2004).

5.4 These studies failed to detect any significant changes in mutation rates in the germ lines of exposed families. This failure may be attributable to: (1) small sample size; (2) a high proportion of families contained irradiated mothers and non-irradiated fathers; (3) successful repair of radiation-induced damage over a long period time prior to conception; (4) a truly low induced mutation frequency.

Populations in the heavily polluted rural areas around the Chernobyl nuclear plant

5.5 Three publications have presented analyses of minisatellite mutation rates in post-Chernobyl families from rural areas of Ukraine and Belarus (Dubrova et al, 1996; 1997; 2002b). In the Belarus study (Dubrova et al, 1996; 1997):

- 127 children of irradiated parents from the Mogilev region of Belarus and 120 children of non-exposed Caucasian parents from the UK were analysed;
- Control and exposed groups were not matched;
- Germline mutations were scored using two multi-locus and eight single-locus probes that detect the most unstable human minisatellite loci.

5.6 The results of this study indicated that mutation rates in the Belarus cohort were elevated for most of the minisatellite loci. Moreover, within the exposed cohort, the mutation rate was significantly greater in families with higher parental radiation dose estimated for chronic external and internal exposure to caesium-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 that this dose reflects only one, and perhaps a minor, component of human radiation exposure after the Chernobyl accident. Given that mutation rates in the exposed group were compared with those in the non-exposed Caucasian families of different ethno-geographic origin, the results of this study do not provide sufficient evidence to support radiation-induction of the germline mutations seen.

5.7 To verify the results of this study, minisatellite mutation rates were evaluated in exposed and non-exposed families from rural areas of the Kiev and Zhitomir regions of Ukraine (Dubrova et al, 2002b). In this study:

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

5.8 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 germline of exposed mothers. The similarity in mutation rates in the germline of exposed and non-exposed 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, which 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 initial high 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 consistent with the estimates of the doubling dose for germline mutation in humans (UNSCEAR, 2001; Sankaranarayanan and Chakraborty 2000).

The families of Chernobyl clean-up workers

5.9 In three recent studies, the frequency of minisatellite mutation was analysed in the offspring of Chernobyl clean-up workers from the Ukraine (Livshits et al, 2001; Slebos et al, 2004) and Estonia (Kiuru et al, 2003). In all studies, the effects of paternal exposure to ionising radiation were analysed. In the Livshits et al (2001) study:

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

5.10 In the Slebos et al (2004) study:

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

5.11 In the Estonian study:

- 148 children of irradiated parents and 155 children of non-exposed parents were analysed.
- The control group consisted of children conceived by the same parents before the Chernobyl accident.
- Germline mutations were scored using the same eight single-locus probes used by Dubrova et al (1997; 2002a,b).

5.12 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 (Pitkevitch et al, 1997). Importantly, the group of Chernobyl clean-up workers was exposed to repeated small daily doses of ionising radiation. The relatively low-dose exposure of these Chernobyl clean-up workers (mean dose 0.11 Sv, see Kiuru et al, 2003), suggests that the expected increase in mutation rate in this group may be too small to detect. The Slebos et al (2004) study did however detect a modest increase in microsatellite mutation frequency in children of exposed fathers; the increase did not however reach statistical significance. The results of a more recent study show a lack of any measurable increase in the microsatellite mutation rate in the germline of Chernobyl clean-up workers (Furitsu et al, 2005).

5.13 A further study reported a seven-fold increase in mutation rate in a cohort of 42 children of 'liquidators' involved in the immediate response to the Chernobyl incident (Weinberg et al, 2001). In the study, Weinberg et al used short random-sequence PCR primers to amplify DNA segments from the human genome (a non-validated technique for monitoring germline mutation in humans) (see Jeffreys and Dubrova, 2001). Given that the doubling dose for mammalian germline mutation has been estimated as 1 Gy (UNSCEAR, 2001), such an increase would imply that the studied cohort has been exposed to doses of up to 6 Gy, a whole body dose that is generally lethal. In fact, most participants in the decontamination work around the Chernobyl nuclear power plant, sarcophagus construction and other clean-up operations received doses of external exposure of less than 0.25 Gy (Pitkevitch et al, 1997). Clearly, these findings contrast with the negative results of three large studies on TRDL mutation rates in Chernobyl clean-up workers (Livshits et al, 2001; Kiuru et al, 2003; Slebos et al, 2004; Furitsu et al, 2005).

Nuclear weapon tests and minisatellite mutation rates

5.14 Minisatellite mutation rates were also evaluated in the germline of irradiated parents living around the Semipalatinsk nuclear test site (Dubrova et al, 2002a). Semipalatinsk has been 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 mainly exposed 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:

- The exposed group comprised 40 three-generation families inhabiting the rural areas of the Semipalatinsk district of Kazakhstan around the Semipalatinsk nuclear test site.
- The control group was composed of 28 three-generation non-irradiated families from the geographically similar rural area of the former Taldy Kurgan district of Kazakhstan, which was not contaminated by nuclear tests.
- Control and exposed groups were matched by ethnicity, maternal age, parental occupation and smoking habit.
- Germline mutations were scored employing the same eight single-locus probes used by Dubrova et al (1997; 2002b).

5.15 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 ionising radiation following the decay of radioisotopes in the late 1950s after the cessation of surface and atmospheric nuclear tests, a negative correlation between 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 radiation-induced.

Cancer chemotherapy and radiotherapy patients

5.16 A novel small-pool PCR (SP-PCR) approach for the detection of minisatellite mutations has been used to analyse mutation induction in the germlines of male cancer patients (Armour et al, 1999; May et al, 2000; Zheng et al, 2000). 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* mutants in a single male (Jeffreys et al, 1994). 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 (Jeffreys et al, 1994; Buard et al, 1998; Tamaki et al, 1999), effectively precluding comparisons of mutation rates between non-exposed and exposed 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, and which are capable of causing subsequent mutations. 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.

5.17 Using SP-PCR of the MS205 minisatellite, germline mutation rates were determined in cancer patients treated with therapeutic mutagens (Armour et al, 1999; Zheng et al, 2000). In the first study, sperm samples from two men, collected before and after treatment with the anticancer drugs cyclophosphamide, etoposide and vincristine, were analysed and no effects on the minisatellite mutation rate were detected (Armour et al, 1999). The alkylating agent cyclophosphamide induces a variety of germ cell effects in post-meiotic stages only, with no effects on pre-meiotic stages (Witt and Bishop, 1996). The topoisomerase-II inhibitor etoposide affects only meiotic germ cells and is not mutagenic in pre- and post-meiotic cells (Russell et al, 1998; Vilarino-Guell et al, 2003). If correct, then the window of time for mutation induction by this drug is very short and may be difficult to analyse. Vincristine prevents the assembly of tubulin into spindle fibres and there is no indication of germ cell mutagenicity for this drug in mice (Witt and Bishop, 1996). Therefore, the two men analysed in the above-mentioned study (Armour et al, 1999) were exposed to the anticancer drugs, which, because of their 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 of time.

5.18 In the second study, sperm samples from ten men collected before and after treatment for Hodgkin's disease were analysed (Zheng et al, 2000). Nine patients, treated either with vinblastine or

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 (Witt and Bishop, 1996). Adriamycin is an intercalating agent and an inhibitor of topoisomerase-II. Exposure to adriamycin predominantly results in cell toxicity in mouse germ cells, without mutation induction (Witt and Bishop, 1996). Bleomycin, a radiomimetic drug, selectively targets mouse oocytes; mutation induction has not been observed in male germ cells (Witt and Bishop, 1996). 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 (Witt and Bishop, 1996), showed a significant increase in mutation rate after chemotherapy (Zheng et al, 2000).

5.19 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 (May et al, 2000). 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 (UNSCEAR, 2001; Sankaranarayanan and Chakraborty 2000).

Conclusions from human studies

5.20 The analysis of minisatellite loci potentially can provide a system for monitoring germline mutation in humans. The main advantage of this system is the ability to detect changes of mutation rates in relatively small population samples, which is attributed to the very high spontaneous instability of these loci. Using this system the first experimental evidence was obtained that the mutation rate in a human population may be increased following ionising radiation exposure. However, to date the human experimental data have been derived from relatively small numbers of families, so 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 have 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 other environmental monitoring studies - for example exposure to chemical contaminants in addition to radiation (see 8.2). Studies showing elevated mutation rate in irradiated families from Belarus, Ukraine and Kazakhstan (Dubrova et al, 1996, 1997, 2002a, 2002b) 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.

Table 4. Summary of published studies on germline ESTR mutation in mouse, modified from Niwa (2003)

Male mice	Female mice	Radiation type; dose (dose rate)	Mating weeks post-irrad	Locus/probe	Main Observations
^a 101/HY x C3H/SnY	101/HY x C3H/SnY	γ -ray; 0.5, 1 Gy	6	Multi locus/33.6, 33.15	1.5-1.9x increased mutation rate doubling dose 0.5Gy
^b C3H/HeN	C57BL6N	γ -ray; 3 Gy (0.5 Gy/min)	0-1, 2-3, 10-11	Single locus/Mh6-hm	Spermatids sensitive
^c C3H/HeN	C57BL/6N	γ -ray; 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
^d C3H/HeN	C57BL/6N	Neutron; 0.35, 0.7, 1 Gy (0.006 Gy/min)	0, 2, 10	Single locus/Mh6-hm	RBE of 2.6 spermatid
^e CBA/H	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
^f C57BL/6 x CBA/H	CBA/H	X-rays; 1 Gy (0.5 Gy/min)	3, 4, 5, 6	Multilocus/MMS10; single locus/Mh6-hm, Hm2	Post meiotic stage insensitive
^g CBA/H	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
^h C57BL/6N	C3H/HeN	γ -ray; 6 Gy (0.5 Gy/min)	0-1	Single locus/Mh6-hm	Maternal allele mutated by spermatozoa irradiation
ⁱ CBA/H,C57BL & BALB/c	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
^j CBA/H,C57BL/6, BALB/c & C.B17	CBA/H,C57BL/6, 129SVJ & 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)

^aDubrova et al, 1993 ; ^bSadamoto et al, 1994 ; ^cFan et al, 1995 ; ^dNiwa et al, 1996 ; ^eDubrova et al, 1998 ; ^fBarber et al, 2000 ; ^gDubrova et al, 2000a ; ^hNiwa and Konminami, 2001 ; ⁱBarber et al, 2002; ^jDubrova, 2005.

6. Radiation-induction of ESTR mutation in germ cells of male mice

6.1 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 (Dubrova et al, 1993; Sadamoto et al, 1994). 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 (Kelly et al, 1991). 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 towards increases in size (Niwa and Kominami, 2001; Yauk et al, 2002). Most studies have used the same specific sequences (the 5 base-pair repeat *Ms6-hm* on mouse chromosome 4, and the 4 base-pair repeat *Hm-2* mapping on 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 (because of their relatively small size, representing about 1 part in 200,000 of the total mammalian genome), it was concluded that mutations arise from some form of radiation-induced genetic instability leading to non-targeted mutational events (Sadamoto et al, 1994; Fan et al, 1995; Dubrova et al, 1998).

6.2 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-ionising 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 summarises the published studies and their conditions (Niwa, 2003).

Dose responses, dose rate effects and doubling doses

6.3 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 (Fan et al, 1995; Dubrova et al, 1998; Dubrova, 2005). While average mutation rates increased by 3-4 fold 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 (3-4 fold) at a given dose. Similar data were reported for more densely-ionising neutron irradiations; where sufficient data have been reported, the relative effectiveness of fission neutrons was 2.6 (for spermatid irradiation; Niwa et al, 1996) or 3.4 for spermatogonial stem cells (Dubrova et al, 2000a).

6.4 Generally, using conventional mutation measurements, the risk of genetic effects of sparsely-ionising 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 (Russell and Kelly, 1982). However, for the induction of ESTR mutation in spermatogonial stem cells, γ -irradiation 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 (Dubrova et al, 2000a). 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 (Russell and Kelly, 1982). Thus, Dubrova et al estimated the doubling dose to be around 0.5 Gy for pre-meiotic stage irradiation and 0.33 Gy in their later studies (Dubrova et al, 1993; Dubrova et al, 1998; Russell and Kelly, 1982; Dubrova et al, 1998). Estimations were also made by Niwa et al, but the doubling dose of 0.8 Gy for spermatid irradiation was the only value based on significant data (Fan et al, 1995). Most recently, Dubrova (2005) has estimated X-ray doubling doses for pre-meiotic cells varying between 0.4 and 1.0 in 5 different strains of mice; this variation was not due to differences in mutation induction in the different strains (see below), but to variation in spontaneous mutation rates. Standard errors on doubling doses are close to 50% (Dubrova, 2005) based on the assumption of a linear dose response.

The controversy over the mutability of different mouse germ cell stages

6.5 In these ESTR mutation experiments, male mice were irradiated and mated at different times afterwards to unirradiated female mice; depending on the time of mating, the effects of irradiation 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) (Adler, 1996). 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 post-irradiation for both dominant visible and recessive lethal mutations (Russell et al, 1998).

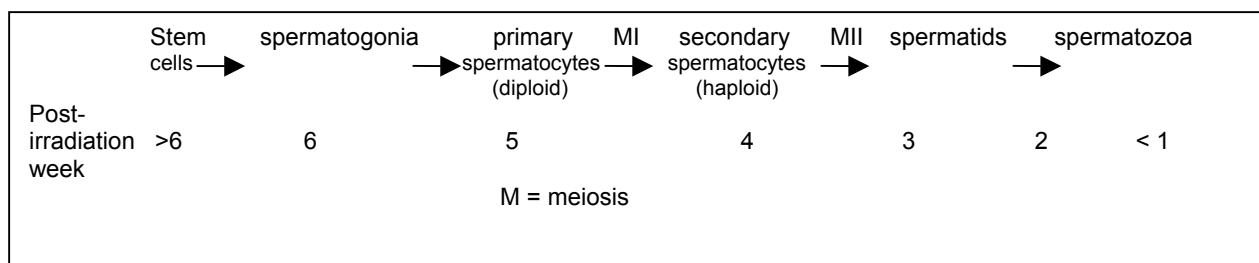


Fig 1 Stages of mouse male germ cell development and approximate post-irradiation mating times that reveal effects on specific stages

6.6 Germ cell stage specificity for ESTR mutation was first reported by Niwa and colleagues (Sadamoto et al, 1994; Fan et al, 1995), irradiating C3H/HeN mice with up to 3 Gy γ -rays, followed by mating to C57BL/6N females either within one week (assessing spermatozoa), or at 2-3 weeks (spermatids), or at 10-11 weeks (stem cells). Only spermatid irradiation gave 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 but this rate was lower than for spermatids (Niwa et al, 1996). These ESTR data therefore tended to agree approximately with data on stage specificity using conventional gene mutation measurements.

6.7 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 (Dubrova et al, 1998; Dubrova et al, 2000a). 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 premeiotic stem cells (6 week matings) and spermatogonial stem cells (10 week matings), whereas no increase was found for spermatid irradiations (3 week matings). In a further study, however, these workers 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 (Barber et al, 2000).

6.8 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. While a large range of strains has not been tested, it is noteworthy that recent studies with up to 5 commonly-used mouse strains showed similar ESTR mutation induction patterns in pre-meiotic germ cells (Barber et al, 2002; Dubrova, 2005). 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 (Russell et al, 1991). Loss of protamine in mice has been found to cause severe DNA damage during condensation and packaging of DNA in the sperm head (Cho et al, 2003). Experiments designed to re-examine the consequences of irradiation of well-defined spermatid stages may help to resolve these discrepancies.

6.9 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 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 co-workers (Sadamoto et al, 1994; Fan et al, 1995), but it is unclear whether a similar criterion has been used in other laboratories. However, use of a different method for assessing ESTR mutations, viz, amplifying 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 (Yauk et al, 2002). 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 showing 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 give progeny for analysis. Additionally, in some studies it is not clear the extent to which somatic mutations (revealed by mosaicism) are included in the scores.

7. Transgenerational Mutation Studies

7.1 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 fathers have been considered. It is however likely that many of these mutations are occurring during sperm cell maturation in the irradiated father and are thus not transgenerational in the strict sense defined in the introduction - ie, that transgenerational mutations are those occurring in the cells of descendants of an irradiated parent. Mutations which 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

7.2 Early indications of transgenerational effects were seen in the experiments of the Niwa group (Sadamoto et al, 1994; Fan et al, 1995) and Dubrova et al (2000a,b). These have been more comprehensively followed up 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 (≥ 2 -fold) when the offspring were derived from immediate matings (spermatozoa irradiated), but showed no such increase when derived from 15-week matings (stem cells irradiated) (Niwa and Kominami, 2001). The lack of mutation induction in maternally-derived ESTR sequences following premeiotically-irradiated germ cell matings had also been established in earlier studies (Dubrova et al, 1998). Nonetheless a trans-acting process has been demonstrated in mice where a reporter gene was activated upon fertilisation by irradiated sperm (Shimura et al, 2002). 7.3 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 6-fold increase in ESTR mutation rate in the germline of their non-exposed offspring. If the male and female offspring were mated further to unirradiated mice, it was found that the ESTR mutation rate was still elevated about 6-fold in males and about 3.5-fold in females (Dubrova et al, 2000a). These next-generation increases were in part derived from mutations that arose after fertilisation, 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 ($p = 0.044$) more frequent in male second-generation offspring (but not in females; $p = 0.13$). In more extensive experiments, the same workers demonstrated trans-generational ESTR mutation rate increased for 2 generations following both X-ray (2 Gy) and neutron (0.4 Gy) irradiations of different strains of mice (Barber et al, 2002). These multi-variable experiments are summarised in Table 5 (data for spermatogonial irradiation only), showing little or no reduction in the persistence of elevated ESTR mutation rate even after two generations, irrespective of mouse strain or radiation type.

7.4 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 (Barber et al, 2002). 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.

Transgenerational mutation at other loci

7.5 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 (Gondo et al, 1993). The p^{un} locus is highly unstable, 1.8% or more of offspring are mosaic revertants (Melvold 1971). However, reversion frequency is variable and can be as high as 12.2% (Aubrecht et al, 1999), thus 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 be observed (Brilliant et al, 1991). Somatic reversion is proposed to proceed by homologous recombination (Gondo et al, 1993).

7.6 Weak evidence indicative of transgenerational effects on p^{un} reversion following irradiation of male p^{un}/p^{un} mice has been reported (Carls and Schiestl, 1999). 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 there may be a transmissible elevation in reversion frequency. However, the data provided in this paper alone are insufficient to have 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) following male germ cell irradiation (Shiraishi et al, 2002). Assessment of reversion in RPE is considered to be more accurate than the fur spot assay (Bishop et al, 2000, 2001).

Table 5 Radiation induction of transgenerational mutation of ESTR sequences (Barber et al, 2002)

Mouse strain	Irradiation	Generation*	Mutation rate**	Rate ratio (to unirradiated)
CBA/H	None	-	0.07	-
		0	0.21	2.9
		1	0.25	3.5
	X-rays	0	0.19	2.6
		1	0.22	3.1
		2	0.24	3.3
C57BL/6	None	-	0.06	-
		0	0.27	4.2
		1	0.16	2.4
		2	0.15	2.4
BALB/c	None	-	0.12	-
		0	0.28	2.4
	X-rays	1	0.31	2.6
		2	0.25	2.1

* 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.

** calculated as the number of mutations/number of offspring per locus (since mutations in controls could originate from either parent, the frequency is divided by 2 for comparison with radiation-induced frequencies where mutations originate from the male only).

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. 6 Gy x-irradiation 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.

7.7 The medaka fish has also been used for studies of transgenerational mutation using a specific locus test (eg, Shimada and Shima, 1998). 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 as it has a high spontaneous mutation frequency, in this respect the locus may be analogous to p^{un} in the mouse. A gamma-radiation dose-dependent induction of whole-body and mosaic wl mutants has been observed (Shimada and Shima, 1998). Mosaics, which are indicative of a transgenerational mutation, were more readily induced 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 - ie, were delayed. More recent studies (Shimada and Shima, 2004) 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 fertilisation - no elevated mutation was detected in male F2 offspring from an irradiated male. These findings are similar to those of Shirashi et al (2002) using the p^{un} RPE system.

7.8 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

7.9 There are few data available from humans which 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 (Dubrova et al, 2002). In this study, no clear evidence of transgenerational effects at minisatellite loci over two generations was reported; effects were only noted in offspring of parents known to have been themselves 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.

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

8. Environmental Monitoring Studies

8.1 The majority of reports concerning radiation-associated changes in mutation rate 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 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.

8.2 Two significant problems face studies of this nature. First, accurate radiation dosimetry is usually very difficult to obtain and consequently dose estimates are at best poor and at worst absent. Secondly, environments such as the Chernobyl site have also been contaminated with heavy metals and other pollutants as well as radiation (Hillis, 1996). Studies of human populations in contaminated areas face similar problems. Thus, environmental monitoring studies are unlikely to provide

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 and mice exposed to pollutants other than radiation in highly industrialised regions (Yauk and Quinn, 1996; Somers et al, 2002, 2004). 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 (Somers et al, 2002). A follow up study was conducted in which mice were housed in HEPA-filtered cages or non-filtered cages at either the polluted or control sites (Somers et al, 2004). In this way it was demonstrated that particulates of greater than 0.3 μm diameter were the major contributor to elevated germline mutation frequencies (Somers et al, 2004).

8.3 Table 6 summarises 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 where a six-fold 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.

9. Mechanisms of tandem repeat DNA locus mutation

Background

9.1 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. While much of the damage to cells is processed rapidly by cellular repair enzymes (ie, within a few hours of irradiation), there is evidence that cellular responses occur over much longer periods.

9.2 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 tumours, and showed that radiation caused high-frequency events that predisposed the cells towards later (spontaneously-occurring) changes (Kennedy and Little, 1984; Kennedy et al, 1984). In follow-up experiments, it was found that the mutation frequency in inessential genes such as *HPRT* was persistently increased in a large fraction of clones surviving irradiation (Chang and Little, 1992). 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 (Little, 1994).

9.3 Chromosomal aberrations have also been found to persist following 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 post-irradiation. The relatively high frequency of aberrations, especially for neutrons, and the occurrence of chromatid-type aberrations on the third mitosis following irradiation suggested that new aberrations were being produced in post-irradiation cell cycles (Weissenborn and Streffer, 1988a; Weissenborn and Streffer, 1988b). Genetic instability in clones of cells surviving irradiation has also been described for alpha-particle irradiation of cultured haematopoietic stem cells from CBA/H mice. In this case, chromosomal aberrations were measured in cell clones surviving 3 Gy from X-rays or 0.25-1 Gy from alpha particles (0.5 Gy from ^{238}Pu alpha particles corresponds to an average of one track per cell). About 50% of the clones surviving alpha-particle irradiation carried aberrations; these were mostly chromatid-type aberrations, suggesting that they had arisen many generations after irradiation (Kadhim et al, 1992).

Table 6. Summary of Chernobyl-zone molecular mutation studies

Species	Reference	Locus	Dose/dose rate	Effect	Comments
Lab mice - C57BL/6 and BALB/c	Wickliffe et al, 2003	Mitochondrial cytochrome b	Cumulative doses of 1.2-1.6 Gy at ~0.04 Gy/day	No significant elevation in mutation rate post-exposure	Criticised on technical grounds by Dubrova (2003)
Barn swallows <i>Hirundo rustica</i>	Ellegren et al, 1997	Microsatellites <i>HrU6, HrU9</i>	No dosimetry - difficulties with migration etc	<i>HrU6</i> : higher (10 fold or more) mutation frequency in Chernobyl zone birds compared to local and distant (Italian) control zone birds. <i>HrU9</i> : 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 <i>Triticum sp</i>	Kovalchuk et al, 2000	13 single copy microsatellites	Contaminated plots - 900 Ci km ⁻² , 0.3 Gy (2/3 external) control plots - <1 Ci km ⁻²	~6 fold elevation in mutation rate in contaminated plot grown plants	Mutation rate in control plot grown plants also high, 1.03 x 10 ⁻³ per locus

9.4 Chromosomal instability in bone marrow cells was also found to be transmissible *in vivo*, by transplanting male cells irradiated with alpha particles into female recipients (Watson, Lorimore and Wright, 1996; Watson et al, 2001). The repopulated haemopoietic system showed instability persisting for up to one year. Alpha particles have also been shown to induce similar delayed chromosomal effects in the bone marrow of two out of four normal humans (Kadhim et al, 1994). 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 (Griffin et al, 2000; Bouffler et al, 2001; Dugan and Bedford, 2003). In an attempt to link the delayed appearance of chromosomal aberrations in cultured mammary epithelial cells to cancer-proneness, Ponnaiya et al (1997) 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.

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

Enhanced mutation at ESTRs

9.6 The high levels of mutation at ESTRs display some similarities with the events described above, but it is not clear whether related mechanisms are involved. Therefore we will discuss candidate mechanisms as well as outlining some approaches that may help us to understand the mechanism(s) behind such events, in the hope that these will promote further discussion and experimentation.

9.7 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 a hundred-fold) than could possibly be explained by the amount of DNA damage we know occurs following specific radiation doses. How then can this happen?

9.8 It must be assumed that some radiation-induced event triggers the ESTR sequences to become genetically less stable, but that this primary event does not occur at these sequences. Well-documented pathways leading to mutation involve mis-replication 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.

9.9 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 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 two metres of DNA, yet it is packaged into chromatin so as to fit into the microscopic human cell. While the DNA has to be packaged precisely, access must be provided to ensure that the cell can carry out its functions (eg, retrieve 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 remodelling factors, including proteins that can covalently modify histones by the addition or removal of acetyl, phosphate or methyl groups (Peterson and Laniel, 2004). These histone modifications change the extent to which DNA is bound to the nucleosomes, influencing the ability of exogenous agents to damage DNA (Mateos et al, 1998) and the ability of damage-response proteins to access DNA. Specific combinations of the various chromatin

remodelling factors, making up a pattern or 'code', are considered to be responsible for controlling access to specific regions of the genome.

9.10 In this context, it could be that access to ESTRs is suppressed by a specific histone code, and that changes to this 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 (eg, genes encoding acetylases, deacetylases, kinases (which add phosphate groups), phosphatases (which take off phosphate groups), methylases and demethylases, etc). It is also possible that the chromatin code differs for highly repetitive sequences between mouse and man. 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 metabolised in mice versus humans, since the chromatin code in the respective regions where the ESTRs reside may well be different.

9.11 A number of authors have suggested that epigenetic changes may be involved in transgenerational ESTR mutation (Dubrova et al, 2000a; Barber et al, 2002; Niwa, 2003). Epigenetic modifications will alter DNA function without changing the sequence; one example is that of genes which may be silenced through the methylation of specific DNA bases in their regulatory sequences. However, 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 colour gene (Morgan et al, 1999), then for an allele of the *Axin* gene involved in embryonic axis formation (Rakyan et al, 2003). 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 only found 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 fertilisation.

9.12 In the development of human cancers, it is known that methylation of tumour suppressor genes can be a mechanism for somatic loss of gene expression ('epimutation'), but recently individuals with multiple cancers have been found with germline epimutation of the DNA mismatch repair gene *MLH1* (Suter et al, 2004). The epimutations were the result of persistent hypermethylation of the promoter region of the gene, again consistent with incomplete erasure of DNA modifications in the germline.

How can we unravel what is happening?

9.13 The isolation of mutant organisms that show altered levels of response has been a very valuable means of understanding how damage-response pathways operate. Characterisation of these mutants ultimately leads to identification of which gene(s) are altered to give the difference in response. Once a gene is identified and its DNA sequence determined it is often possible to determine what its product (an enzyme or a structural protein) does 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.

9.14 Mutants may be used from a variety of organisms, ranging from unicellular bacteria and yeasts, where it is much easier and quicker to find and analyse 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 base pairs) 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 (Debrauwere et al, 1999). Similar data were obtained for trinucleotide repeats in yeast (Jankowski et al, 2000). 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 (Jauert et al, 2002). Further, while minisatellites are normally stable in 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) (Lopes et al, 2002). Altogether these data show that minisatellites in yeast may mimic several of the characteristics of human minisatellites, that DNA breaks are important to generate instability, and that nucleases involved in removing additional sequence generated in 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 at present, it seems likely that homologous recombination is involved at some stage of the process (Jauert et al, 2002; Lopes et al, 2002). 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.

9.15 We have also summarised above the evidence for the transmission of a hypermutable state at ESTR loci in mice. It is worth noting that some years ago Fabre and Roman (1977) 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 unirradiated egg in the mouse experiments. Following ideas initially proposed by Holliday (1971), Fabre and Roman suggested that radiation caused the release of some factor(s) that normally repress recombination. Fabre followed this up with further (unpublished) data describing a radiation-induced recombination mechanism that is transmissible 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.

9.16 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 (2000) compared crossover frequency with germline ESTR mutation frequency following 1 Gy X-rays. They found an approximately 3-fold increase in ESTR mutations, but no general change in crossover frequencies measured at 25 sites on 6 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).

9.17 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 following irradiation of male germ cells (Barber et al, 2002; Niwa and Kominami, 2001) suggests that the mutational mechanism operates in the zygote following fertilisation, where paternal and maternal nuclei share a common environment. Interestingly, a trans-acting process has been found in mice where a reporter gene was activated upon fertilisation by irradiated sperm (Shimura et al, 2002). 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 following irradiation (Yauk et al, 2002). 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 at these two stages.

10. Potential implications of minisatellite mutation for human health

Minisatellite expansion and contraction in human disease

10.1 Since minisatellite mutation events occur in both 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 length to alter gene coding sequences (Denoeud et al, 2003), influence gene expression (Kominato et al, 1997; Prokhortchouk et al, 1998) or generate fragile sites (Handt et al, 2000). For example, a minisatellite upstream of the cystatin B (*CSTB*) gene is normally present in 2 or 3 copies but is expanded to 30-80 copies in individuals with a specific form of epilepsy (Virtaneva et al, 1997; Lalioti et al, 1997). The expanded alleles exhibit a greatly increased mutation rate (47%) (Larson et al, 1999) and give rise to a dramatically reduced level of *CSTB* gene expression (Alakurtti et al, 2000).

10.2 The *INS* minisatellite has been shown to influence the transcription of both the *INS* gene (Kennedy et al, 1995) and insulin-like growth factor 2 (*IGF2*) gene (Paquette et al, 1998) and certain genotypes may be associated with a number of different conditions/phenotypes including insulin-

dependent diabetes (Vafiadis et al, 1997; Pugliese et al, 1997), insulin sensitivity and secretion (Bazaes et al, 2003), childhood obesity (Le Stunff et al, 2001), birth size (Dunger et al, 1998) and polycystic ovary syndrome (Waterworth et al, 1997).

10.3 Homozygosity for a particular allele (L) of the MNS16A minisatellite, located downstream of the human telomerase (*TERT*) gene, has been associated with a two-fold increased risk of lung cancer (Wang et al, 2003), whereas rare alleles of the *HRAS* minisatellite may be associated with increased risk of a variety of cancers including leukaemia (Krontiris et al, 1993), lung (Lindstedt et al, 1999), breast (Gosse-Brun et al, 1999), brain (Vega et al, 2001), colorectal (Gosse-Brun et al, 1998), ovarian (Weitzel et al, 2000) and bladder (Krontiris et al, 1993). However, their role as a risk factor in tumorigenesis is still somewhat controversial (Langdon and Armour 2003).

10.4 Rare alleles of the *HRAS* minisatellite have also been noted in tissue from spontaneously aborted human embryos (Kiaris et al, 1995), but the results of this study must be open to question owing to the lack of proper controls. Some doubt has recently been cast on the conclusions drawn by the original association studies. Whilst 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 (Langdon and Armour, 2003). The proposed association between rare alleles of the *HRAS* VNTR and various cancers remains therefore to be confirmed or refuted by additional studies.

10.5 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, 1998). It must be realised, 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 can be detected in individuals with the disease but its presence does not cause the disease.

10.6 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 (Wahls et al, 1990) and are also capable of stimulating homologous recombination *in vitro* (Boán et al, 1998). However, minisatellite instability may not be an intrinsic property of the tandemly repetitive DNA, but 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 localised meiotic recombination [hotspots] in the human genome' (Jeffreys et al, 1998).

10.7 Minisatellite core sequences have been reported in association not only with oncogenic chromosomal translocation breakpoints (Wyatt et al, 1992; Krowczynska et al, 1990; Jaeger et al, 1993; 1994; Panagopoulos et al, 1997) but also with the breakpoints of gross deletions causing inherited disease (Lopez-Correa et al, 2001; Lee et al, 2002; Pan et al, 2003). In a recent meta-analysis of translocation breakpoints in human inherited disease and cancer, the minisatellite core element was found to be significantly over-represented (Abeysinghe et al, 2003). Such sequences may represent a distinct class of recognition element for V(D)J recombinase which functions to generate antibody diversity for the immune system (Davila et al, 2001).

10.8 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 worth areas of investigation.

10.9 Finally, there are some reports of sequence similarities between human minisatellites and viral sequences which 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 (Zolotukhin et al, 2001). 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 (Fujiwara and Ono 1995).

11. Conclusions

11.1 TRDL mutation can be used as a biomarker of radiation exposure with mixed success. TRDL mutations have been seen in residents of regions contaminated following the Chernobyl accident and other populations predominantly exposed to internal emitters. However, studies with predominantly external radiation exposure (eg, Chernobyl liquidators, radiotherapy patients, A-bomb survivors) are negative. These studies may be hampered by small sample sizes and inaccurate radiation dosimetry. It is notable that the positive studies involve low dose exposures, mainly from internal emitters.

11.2 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 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 mutations occur distant to direct DNA damage targets.

11.3 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.

11.4 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 understanding of ESTR mutation were outlined in section 9.

11.5 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, 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, a generalisation of potential health effects of TRDL mutation should not be made at the present stage of our understanding.

11.6 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 risk estimates. For cancer and other conditions however, these mutations may play a role and only further research will determine this.

11.7 We have no knowledge as to whether TRDL mutations might influence pregnancy outcome, development or viral disease. Interactions between TRDL mutations and viral sequences cannot be excluded.

11.8 Whether CAT scans or other medical diagnostic and therapeutic procedures can induce TRDL mutations is unknown.

12. Suggested areas for further investigation

12.1 It will be apparent to readers of this report that there are many uncertainties and gaps in our knowledge. The suggestions below are wide-ranging and not necessarily comprehensive. Government and research funding agencies must make judgements on priorities.

12.2 A considerable effort should be made to extend the analysis of minisatellite mutation induction in humans to other cohorts of irradiated families, including:

- a) the offspring of families of patients of childbearing age treated with radiation for therapeutic and diagnostic purposes including CAT scans. Sperm samples could also be taken before and after radiation, and the doses received will be known. Comparison might be possible between internal and external exposures

- b) the population exposed to radiation from discharges of radioactive waste into the Techa river, Southern Urals; occupationally exposed families from the Mayak Nuclear Processing Plant; the population from the Gomel district of Belarus exposed to Chernobyl fallout; families of Chernobyl plant personnel and clean-up workers. With these populations, issues will occur with respect to accurate estimations of the radiation doses received and it should be noted that several studies have already been undertaken.

12.3 Consideration should be given to the formation of a centralised 'bio-bank' (blood and DNA samples) of families exposed to different types of ionising radiation. This bio-bank could be used in future studies addressing genetic effects of ionising radiation using novel molecular biological methods.

12.4 In order to establish a dose-response relationship for minisatellite mutation induction, considerable effort should be made to establish the doses of human exposure to ionising radiation from external and internal sources by means of physical and biological dosimetry. We are aware that studies with the Kerala exposed population could contribute in this respect.

12.5 Studies to resolve the differences in stage-specific sensitivities reported for mouse ESTR mutation are required. This may involve inter-strain comparisons.

12.6 Opportunities to study transgenerational mutation in human minisatellites should be sought.

12.7 The role of DNA sequence and chromosome structure in determining transgenerational mutability requires investigation.

12.8 Environmental monitoring studies should provide sufficiently robust radiation dosimetry to facilitate interpretation. The availability of laboratory-based species-specific reference data will also strengthen such studies. At present studies housing laboratory mice in contaminated areas appear to be the most promising.

12.9 Mice carrying mutations in selected pathways of DNA metabolism should be examined for levels of germline ESTR mutation.

12.10 The use of model systems should be further explored to see if non-targeted events are ubiquitous, and to speed up the resolution of the mechanism(s) involved. Studies employing other eukaryotes could provide information rapidly due to the availability of numerous strains harbouring mutations in genes with roles in these processes.

12.11 While the use of mouse ESTRs will yield important information on mechanisms of high-frequency mutagenic change in mammals, these may not mimic high-frequency events in the human germline. For example, mouse ESTRs and human minisatellite sequences do not have the same chromosomal localisation. It is therefore important similar studies are employed on the human germline.

12.12 Efforts should be made towards the identification of further TRDLs in human, mouse and other organisms. The mutational properties of these new loci will need to be investigated.

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GLOSSARY

Expanded Simple Tandem Repeat (ESTR)

One of a class of Tandemly Repeated DNA Loci (see below) commonly used for TRDL mutation detection in mouse. Characterised by 4-10 base pair (bp) repeat units with array sizes of 100 bp - 20 kilo base pairs (kb).

Linkage disequilibrium

A non-random association between specific alleles of specific genes in a population. Alleles of genes in linkage disequilibrium tend to be co-inherited due to reduced recombination between the genes generally as a consequence of close physical proximity.

Locus/loci

A site in the genome at which a genetic effect has been observed. Generally a locus will correspond to a specific DNA sequence at a specific location.

Microsatellite

A class of Tandemly Repeated DNA Locus (see below) characterised by 2-6 bp repeat units in arrays of 10 bp-1 kb. Present in mouse and human but not generally used for germline mutation analysis.

Minisatellite

A class of Tandemly Repeated DNA locus commonly used for TRDL mutation detection in humans. Characterised by 9-60 bp repeat units arranged into arrays of 500 bp-15 kb. Generally sub-telomerically located in human genome but probably randomly distributed in the mouse genome.

Mutation Rate

Number of mutations (generally altered bands in gels) passed on to the next generation by an individual in a sample of mouse/human/other population. Calculated by dividing the total number of mutations scored in the offspring by the total number of parental TRDL bands. This corresponds to mutation frequency per generation.

Pedigree approach

A method for mutation analysis by scoring of mutations in full families (Mother-Father-Child/ren). For TRDL mutation detection, DNA samples are extracted (generally from samples of peripheral blood) and mutations detected by hybridisation with locus specific or multi-locus probes. Mutants are detected by the presence of size variants not present in either parent.

Small pool PCR (SP-PCR)

A method of TRDL mutation detection based on the amplification of multiple diluted aliquots of sperm DNA. The frequency of TRDL mutation is estimated by dividing the total number of mutations by the number of amplifiable DNA molecules. Applicable to mice and humans. In humans, a very high variation in spontaneous mutation frequencies between individuals requires pre/post treatment sampling for adequate interpretation.

Tandemly Repeated DNA Loci (TRDL)

DNA sequences found in mammalian cells perhaps accounting for 10% of the nuclear genome. Repeat units of varying size are arranged into larger arrays with repeats organised head to tail. Major classes include minisatellites, microsatellites and ESTRs which are distinguished on characteristics such as repeat unit size, total array size, array complexity and location in the genome.

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