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MOLECULAR BIOLOGY METHODS IN ASSESSING RADIATION-INDUCED HEREDITARY RISKS IN HUMANS

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Summary

Effort to predict the genetic consequences for humans of exposure to ionising radiation has been one of the most important issues of human genetics over the past 60 years. To date, there has been little experimental knowledge on the genetic risks of human exposure to ionising radiation. Radiation-induced deleterious hereditary effects have not been detected in human populations - not even among the offspring of atomic bomb survivors in Hiroshima and Nagasaki. This does not mean deleterious hereditary effects do not exist in humans, but rather that they are small and/or difficult to detect because the normal incidence of inherited abnormalities is quite high in the human population. Thus, assessment of radiation-induced hereditary risks in humans has been based on the common knowledge of human heredity and on animal experiments. However, recent data have suggested that hyper-variable tandem repeat minisatellite loci provide a useful and sensitive experimental approach for monitoring radiation-induced germline mutations in humans.

In order to investigate the feasibility of the minisatellite mutation screening system in assessing radiation-induced hereditary risks in humans, we examined the amount of hereditary minisatellite mutations among the offspring of Estonian Chernobyl cleanup workers. The men studied received a median radiation dose of 109 mSv while working on the cleanup activities after the Chernobyl accident. We compared the minisatellite mutation rates of 155 children born to 147 Estonian Chernobyl cleanup workers after the accident to those of their 148 siblings born prior to it. In addition, 44 Estonian families, where the father had not been exposed to radiation, composed an additional control group.

In all of these families, the paternity of the children was ascertained by using 5 minisatellite loci (*APOB, HRAS, MCOB19, MCT118*, and *YNZ-22*) in PCR-based analyses. Other 8 minisatellite loci (*B6.7, CEB1, CEB15, CEB25*, *CEB36, MS1, MS31*, and *MS32*) were used for minisatellite mutation screening by using Southern blot-based analyses. In these analyses, the parental origin and germline length change were determined for all detected minisatellite mutations at the eight tested loci. Out of these mutations 107 were *de novo* paternal minisatellite mutations (52, 42, and 13 mutants among children born after the accident, before the accident, and in additional control families, respectively)

and 46 were *de novo* maternal minisatellite mutations (21, 17, and 8 mutants among children born after the accident, before the accident, and in additional control families respectively). In addition, 8 paternal and 6 maternal mutations were detected in children born after the accident to fathers who were exposed to radiation during the Chernobyl cleanup activities in 22 families which had no children born before Chernobyl.

The detected minisatellite mutation rates were statistically tested to be non-significantly increased among children born after the accident when compared with their siblings born prior to it (mutation frequencies of 0.042 and 0.036; OR 1.33, 95% CI 0.80-2.20). When the data were divided into three dose groups, the minisatellite mutation rates were similar among children born before and after the Chernobyl accident to fathers exposed to less than 100 mSv (OR 0.95, 95% CI 0.44-2.05) or to 100-190 mSv (OR 1.14, 95% CI 0.47-2.77). The mutation rate among offspring born after the accident to workers who had received doses of 200 mSv or above was three times higher when compared with their siblings born before the accident (OR 3.00, 95% CI 0.97-9.30). However, no clear linear dose response could be detected in the studied minisatellite mutations (OR 1.00, 95% CI 0.96-1.04). The minisatellite mutation rate was not associated with the father's age (OR 1.04, 95% CI 0.94-1.15) or the sex of the child (OR 0.95, 95% CI 0.50-1.79).

In conclusion, the minisatellite screening system proved an efficient method for detecting germline mutations in a human population. However, the radiation doses to which the studied Estonian men were exposed in this study were so low that the results of this study were consistent with either no effect of radiation on minisatellite mutations or a slight increase at dose levels exceeding 200 mSv.

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Avainsanat Ionisoiva säteily, Perinnöllinen riski, Minisatelliitti lokukset, Ituradan mutaatio, Tshernobyl

Yhteenveto

Säteilyaltistuksen aiheuttaman perinnöllisen riskin arviointi väestössä on ollut yksi ihmisgenetiikan haasteista jo 60 viime vuoden ajan. Toistaiseksi on ollut saatavilla hyvin vähän kokeellista tietoa ihmisen säteilyaltistukseen liittyvistä perinnöllisistä riskeistä, sillä säteilyn aiheuttamia vahingollisia perinnöllisiä vaikutuksia ei ole pystytty osoittamaan edes Hiroshiman ja Nagasakin atomipommitusten uhrien jälkeläisillä. Tämä ei kuitenkaan merkitse sitä, että muista eläimistä poiketen ihmisellä ei esiintyisi säteilyn aiheuttamia vahingollisia perinnöllisiä muutoksia. Ihmisellä esiintyvät säteilyn aiheuttamat perinnölliset muutokset ovat ilmeisesti kuitenkin niin pieniä ja/tai vaikeasti havaittavia, että niiden osoittaminen on ongelmallista ihmispopulaatiossa esiintyvien muiden perinnöllisten poikkeavuuksien varsin korkeiden taustaesiintyvyyksien takia. Tämän vuoksi säteilyn aiheuttamien perinnöllisten riskien arviointi ihmisellä on perustunut yleiseen tietämykseen ihmisen perinnöllisyydestä sekä eläinkokeiden tuloksiin. Viimeaikaiset tutkimustulokset ovat kuitenkin osoittaneet, että peräkkäisiä toistoja sisältävät minisatelliittilokukset ovat käyttökelpoinen ja herkkä lähestymistapa tutkia säteilyn aiheuttamia ituradan mutaatioita ihmisellä.

Tämän työn tarkoituksena oli tutkia minisatelliittimutaatioiden kartoitusmenetelmän soveltuvuutta säteilyn aiheuttaman perinnöllisen riskin arviointiin ihmisellä. Tutkittujen 147 virolaisen perheen isät altistuivat keskimäärin 109 mSv:n säteilyannokselle työskennellessään Tshernobylin onnettomuuden jälkeisissä puhdistustehtävissä. Selvitimme perinnöllisten minisatelliittimutaa tioiden määrän näiden miesten lapsissa. Tutkimuksessa vertasimme minisatel liittimutaatioiden määrää 155 Tsernobylin onnettomuuden jälkeen syntyneellä lapsella ja heidän 148 sisaruksellaan, jotka olivat syntyneet perheeseen ennen Tshernobylin onnettomuutta ja isän altistumista säteilylle.

Isyys varmistettiin kaikissa perheissä käyttäen viittä minisatelliittilokusta (*APOB, HRAS, MCOB19, MCT118* ja *YNZ-22*) PCR- pohjaisissa analyyseissä. Kahdeksan muun minisatelliitti lokuksen (*B6.7, CEB1, CEB15, CEB25, CEB36, MS1, MS31* ja *MS32*) avulla tutkittiin minisatelliittimutaatioiden määrä Southern

blot -pohjaisella menetelmällä. Näissä analyyseissa alleelin vanhempi alkuperä ja toiston pituuden muutos verrattuna vanhempien alleeleihin määriteltiin kaikissa kahdeksassa tutki-tussa lokuksessa havaituissa minisatelliittimutaatioissa. Havaituista mutaatioista 107 oli isältä perittyjä *de novo* -minisatelliitti mutaatiota (52, 42 ja 13 mutaatiota onnettomuuden jälkeen syntyneillä lapsilla, ennen sitä syntyneillä lapsilla ja kontrollilapsilla) ja 46 oli äidiltä perittyjä *de novo* -mutaatiota (21, 17 ja 8 mutaatiota onnettomuuden jälkeen syntyneillä lapsilla, ennen sitä syntyneillä lapsilla ja kontrollilapsilla). Tutkimuksessa havaittiin myös 8 isältä perittyä ja 6 äidiltä perittyä mutaatiota lapsilla, jotka olivat syntyneet Tsernobylin onnettomuuden jälkeen puhdistustöissä säteilylle altistuneille isille 22 perheessä, joissa ei ollut ennen Tsernobyliä syntynyttä lasta.

Havaittujen minisatelliittimutaatioiden frekvenssit eivät olleet tilastollisesti merkitsevästi suurempia onnettomuuden jälkeen syntyneillä lapsilla verrattuna heidän ennen onnettomuutta syntyneisiin sisaruksiinsa (vastaavat mutaatiofrekvenssit olivat 0,042 ja 0,036; OR 1,33; 95 % CI 0,80 - 2,20). Kun aineisto jaettiin kolmeen annosryhmään, Tsernobylin onnettomuuden jälkeen syntyneiden lasten mutaatiofrekvenssi ei poikennut ennen onnettomuutta syntyneiden lasten mutaatiofrekvenssistä alle 100 mSv:n annoksen saaneilla miehillä (OR 0,95; 95 % CI 0,44 - 2,05) eikä myöskään 100 - 190 mSv:n annoksen ryhmässä (OR 1,14; 95 % CI 0,47 - 2,77). Sen sijaan 200 mSv:n tai suuremman annoksen saaneilla miehillä onnettomuuden jälkeen syntyneiden lasten mutaatiofrekvenssi oli kolminkertainen sitä ennen syntyneisiin lapsiin nähden (OR 3,0; 95 % CI 0,97 - 9,30). Isän saamalla säteilynnoksella ei kuitenkaan todettu olevan selvää lineaarista yhteyttä minisatelliittimutaatioiden määrään (OR 1.00, 95 % CI 0.96 - 1.04). Isän ikä (OR 1,04; 95 % CI 0,94 - 1,15) tai lapsen sukupuoli (OR 0,95; 95 % CI 0,50 - 1,79) eivät vaikuttaneet minisatelliittimutaatioiden frekvensseihin.

Yhteenvetona voidaan todeta, että minisatelliittimutaatiot osoittautuivat tehokkaaksi menetelmäksi havaita ituradan mutaatioita ihmispopulaatiossa. Tutkitut virolaiset miehet altistuivat kuitenkin Tshernobylissä työskennessään keskimäärin niin pienille säteilyannoksille, että tutkimuksen tuloksen tulkinta ei ole yksiselitteistä. Havaitut tulokset voidaan yhtä hyvin tulkita niin, että säteilyllä ei ole vaikutusta minisatelliittimutaatioden yleisyyteen kuin niinkin, että minisatelliittimutaatiot lisääntyivät hieman isillä, joiden saama säteilyannos ylitti 200 mSv.

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Contents

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Additional data on maternal mutations and on other control families is presented in the thesis.

List of abbreviations

A	adenine
APOB	apolipoprotein B gene
AD	autosomal dominant inheritance pattern
AR	autosomal recessive inheritance pattern
bp	base pair
Bq	Becquerel
C	cytosine
cDNA	complementary DNA
CI	confidence interval
D	absorbed dose
DD	doubling dose
DNA	deoxyribonucleic acid
DSB	double-strand break
E	effective dose
G	guanine
Gy	gray
H	equivalent dose
HRAS	Harvey rat sarcoma oncogene
HVR	hypervariable region
kb	kilobase
LET	linear energy transfer
LNT	linear-no-threshold dose response
Mb	megabase
MC	mutation component
MIM	Mendelian Inheritance in Man
MVR-PCR	minisatellite variant repeat mapping by PCR
Sv	sievert
OMIM	On-Line Mendelian Inheritance in Man
OR	odds ratio
PCR	polymerase chain reaction
PRCF	potential recoverability correction factor
RBE	relative biological effectiveness
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
SP-PCR	small pool polymerase chain reaction
SSB	single-strand break

STR	short tandem repeat
STRP	short tandem repeat polymorphism
Sv	sievert
T	thymine
U	uracil
VNTR	variable number of tandem repeats

1 Introduction

One of the major challenges for modern genetics is to apply recent advances in mutation research to improve the accuracy of the estimation of hereditary risks for humans. It has long been known that ionising radiation induces both somatic and germinal mutations in a wide variety of biological systems, and the development of radiation genetics began with the discovery of the mutagenic effects of ionising radiation in Drosophila (Muller 1927), maize, and barley (Stadler 1928a, b). It has been recognized that ionising radiation not only increases mutation rates in the exposed somatic and germinal cells but also results in an elevated mutation rate with many cell divisions after the initial irradiation damage (Kadhim et al. 1992; Morgan et al. 1996). Despite convincing evidence for radiation-induced deleterious hereditary effects in experimental animals, there is little experimental evidence for these in human populations. The atomic bombs in Hiroshima and Nagasaki in 1945 and the Chernobyl accident in 1986 exposed large groups of people to non-natural environmental radiation. These events led to a dramatic change in the awareness of genetic risks from radiation exposure. Radiation-induced hereditary mutation was considered an important potential environmental health hazard. It was assumed that radiation-induced hereditary diseases were similar to those occurring naturally as a result of spontaneous germ cell mutations. However, the lack of efficient mutation monitoring systems has resulted in considerable uncertainty concerning the effects of environmental radiation on germline mutations in the human population.

Attempts have been made to predict these effects through genetic risk estimation, in which it is considered that even a low radiation dose can cause an increase in the mutation rate or disease incidence in the human population. The radiation exposure of human populations is usually delivered as small doses at high dose rates (e.g., diagnostic radiology), or are greatly protracted (e.g., continuous exposure from natural and man-made sources) (UNSCEAR 2001). Therefore, in radiation risk estimates, the radiation conditions are generally taken to be low-LET radiation at low doses and low-dose-rate. The risk due to radiation has conventionally been expressed as the expected number of cases of genetic disease above the baseline incidence occurring naturally in the population. However, it has to be kept in mind that induced genetic changes that are incompatible with viability (e.g., deletions or dominant lethal mutations) are not seen in live births. They will add to the already high number of spontaneously occurring genetic changes that are normally eliminated as zygotic deaths, early embryonic losses, or spontaneous abortions.

The germ cell stages most at risk for hereditary effects are the stem cell spermatogonia in males and immature oocytes in females (Searle 1987). However, the detection of changes in the germline mutation rate in human populations is extremely difficult since the spontaneous mutation frequency is very low at most loci. Traditionally used methods for evaluating the mutational load in human populations have been, e.g., the search for newborns with congenital malformations and other genetic diseases, evaluation of mortality and the incidence of cancer among children as well as protein electrophoresis. When these classical methods are used, a large number of samples are required to detect *de novo* mutation or an increase in mutation rates. Therefore, the sensitivity of these approaches for monitoring radiation-induced mutations remains highly uncertain (Sankaranarayanan 1999). For that reason, germline mutation induction in mice is still used as the main source of experimental data for evaluating the genetic risk of human exposure to ionising radiation (Sankaranarayanan and Chakraborty 2000a; UNSCEAR 2001). The induction rates of mutations and chromosomal aberrations in mammalian experimental systems are converted into estimates of risk of radiation-induced hereditary diseases in human populations. Such an extrapolation has not been experimentally validated and is consequently unproven.

It is increasingly clear that reliable estimates of the genetic risk of human exposure to ionising radiation can only be derived from the relevant experimental data on germline mutation induction in human populations. This requires new experimental approaches and there has been a shift from the purely classical approaches to human molecular biology. Molecular techniques have been increasingly used to study radiation-induced germline mutations. Compared with traditional protein analysis, DNA analysis is more informative and it can be done in minute or degraded material. In addition, DNA genotype can be obtained from any tissue whereas the analysis of protein markers is restricted to cells where these proteins are expressed. Very high rates of spontaneous germline mutations altering the length of minisatellite loci has been found in human populations, thus enabling the detection of induced mutation rate in relatively small sample sizes. Germline mutations at tandem repeat minisatellite loci have been shown to be remarkably sensitive to ionising radiation, both in mice (Dubrova et al. 1993, 1998a, b, 2000; Yauk et al. 2002) and in humans (Dubrova et al. 1996, 1997, 2002a, b). The finding that this radiation-induced germline instability in minisatellite mutations persists for at least two generations in mice (Barber et al. 2002) has raised important issues of risk evaluation in humans. However, the lack of reliable approaches for monitoring germline mutation currently presents the main obstacle on how to evaluate the hereditary risk of human exposure to ionising radiation.

Progress in genetic risk estimation will be intimately linked to advances in human molecular genetics and the results of the Human Genome Project, providing important insights into the nature and mechanisms of origin in naturally occurring genetic diseases and their potential to be induced by radiation. Human studies have shown that at low doses, the hereditary risks are likely to be small compared with the baseline risks of naturally occurring genetic diseases. There are several poorly known genetic fenomena (e.g., individual radiosensitivity and genetic instability) that may show to be of ultimate importance of hereditary risk evaluation even for very low radiation doses. On the other hand, as increasing numbers of childhood cancers are successfully treated with high-dose, high-dose-rate irradiation, the need to estimate hereditary risks for these radiation conditions may also become more important in the coming years.

As new methods for radiation risk assessment are potentially needed both in very low and very high doses and as minisatellites currently provide the only loci appropriate for the efficient monitoring of germline mutation in human populations, the present study was designed in order to investigate the potential of minisatellites for monitoring radiation-induced germinal mutations and to examine the applicability of the minisatellite mutation screening system for future demands in evaluating radiation-induced hereditary risks in humans.

In the present work, the hereditary effects of low radiation doses were examined by comparing the minisatellite mutation rates of children born to Estonian Chernobyl cleanup workers after the accident with those of their siblings born prior to it. Correlation was sought between the individual radiation doses of the Estonian Chernobyl cleanup workers and the amount of minisatellite mutations found in their offspring born after the accident. Furthermore, additional information was provided by detecting maternal minisatellite mutations in the same families and control families as well as paternal mutations in control families.

2 Review of the literature

2.1 The human genome

2.1.1 Physical structure of the human genome

The human genome is made up of two components: the nuclear genome and the mitochondrial genome. The nuclear genome consists of approximately 3 300 000 kilobases (3 300 Mb) of deoxyribonucleic acid (DNA). DNA is a double-stranded helix containing a sugar(deoxyribose)-phosphate backbone and four different nucleotide bases: adenine (A), cytosine (C), guanine (G), and thymine (T). DNA strands are linked by hydrogen bonds between A and T and between C and G in opposite DNA strands. Nuclear DNA is arranged in 22 pairs of linear DNA molecules (chromosomes, autosomes) together with an X and Y chromosome (sex chromosomes, XX for females and XY for males). Except for mature red blood cells, each of the approximately 10^{13} cells in the adult human body contains a complete genome. The vast majority of human cells are diploid and they contain two copies of the human genome and 46 chromosomes. These are called somatic cells, in contrast to sex cells or gametes, which are haploid and have 23 chromosomes (one of each autosome and one sex chromosome). The mitochondrial genome is a maternally inherited, circular 16 569 bp DNA molecule. Cells have about 8 000 copies of the mitochondrial genome, about 10 in each mitochondrion.

2.1.2 Organization of the human genome

Since the discovery of the structure of DNA in 1953, knowledge of the composition and organization of the human genome has accumulated rapidly. The Human Genome Project (HGP) started in 1990 and led in April 2003 to the complete, high-quality sequence of the $3.3 \cdot 10^9$ bases that constitute the human genome. However, despite the knowledge of the entire human sequence, the exact number of genes encoded by it is still uncertain. An estimation of the human gene number of about 30 000 genes (Venter et al. 2001) bisects the gene number from the earlier estimate of 60 000-70 000 genes (Fields et al. 1994). Nearly 40% of these predicted 30 000 human genes have already been established (see 2.7.2). The size of human genes varies widely between 0.1 kb and 2 500 kb. An approximate average length of a human gene is 6–10 kb. Each human gene has its own DNA sequence and structure. Most human genes consist of coding (exon) and noncoding (intron) sequences. The average size of a human exon is 180–200

bp with comparatively little length variation. Some exceptionally long exons have been reported (e.g., 7.6 kb exon 26 of the *APOB* gene). Human intron size varies enormously from a few bp to several kb (e.g., 0.5 kb in the β-globin gene and 30.0 kb in the dystrophin gene). Some genes contain a considerable number of introns and in a few human genes (e.g., histone genes, interferon genes, and mitochondrial genes) there are no introns. Venter et al. (2001) detected that only 1.1% of the human genome is spanned by exons. The rest 98.9% of the human genome consists of introns (23.9%) and intergenic DNA (75%) whose functions may include providing chromosomal structural integrity and regulating where, when, and in what quantity proteins are made. The small human mitochondrial genome consists of 37 genes. These include two ribosomal RNA-, 22 transfer RNA-, and 13 polypeptide-encoding genes. The organization of the human genome is presented in Figure 1.

2.1.3 Function of the human genome

The knowledge of the entire human DNA sequence alone does not automatically provide understanding of how each segment contributes to the whole cell or organism. Each DNA molecule (chromosome) contains many genes. In any given tissue, only a relatively small proportion of the genes are expressed. Individual genes represent a single unit of information (specific sequence of nucleotide bases) which must be expressed in a coordinated manner to give life to a cell or organism. Protein-coding genes contain open reading frames (ORFs). The ORF consist of a series of codons that specify the amino acid sequence of the protein that the gene codes for. Upstream from the initiation codon there are a number of noncoding sequences, promoters. Further upstream there are a number of cis-acting regulatory elements of defined sequence. Some of these (TATAAA and CCAAT motifs) play a role in constitutive gene expression. Other regulatory elements (enhancers) increase transcription as a response to particular proteins in a tissue-specific manner. When genes are expressed, the genetic information (base sequence) of DNA is first copied to a molecule of messenger RNA (mRNA) (transcription). RNA is a close chemical relative of DNA. RNA is usually a single-stranded molecule where nucleotide uracil (U) replaces DNA's thymine (T) and sugar is ribose instead of deoxyribose in DNA. The set of mRNA transcripts generated for a cell is called its transcriptome. Both exons and introns are transcribed into precursor mRNA, out of which regions transcribed from introns are later excited and removed (splicing). Therefore, intron sequences do not form mRNA and thus do not specify the primary structure of the gene product. The accuracy of the excision is determined by the invariant GT and AG dinucleotides present at the 5' and 3' exon-intron junctions. An unusual

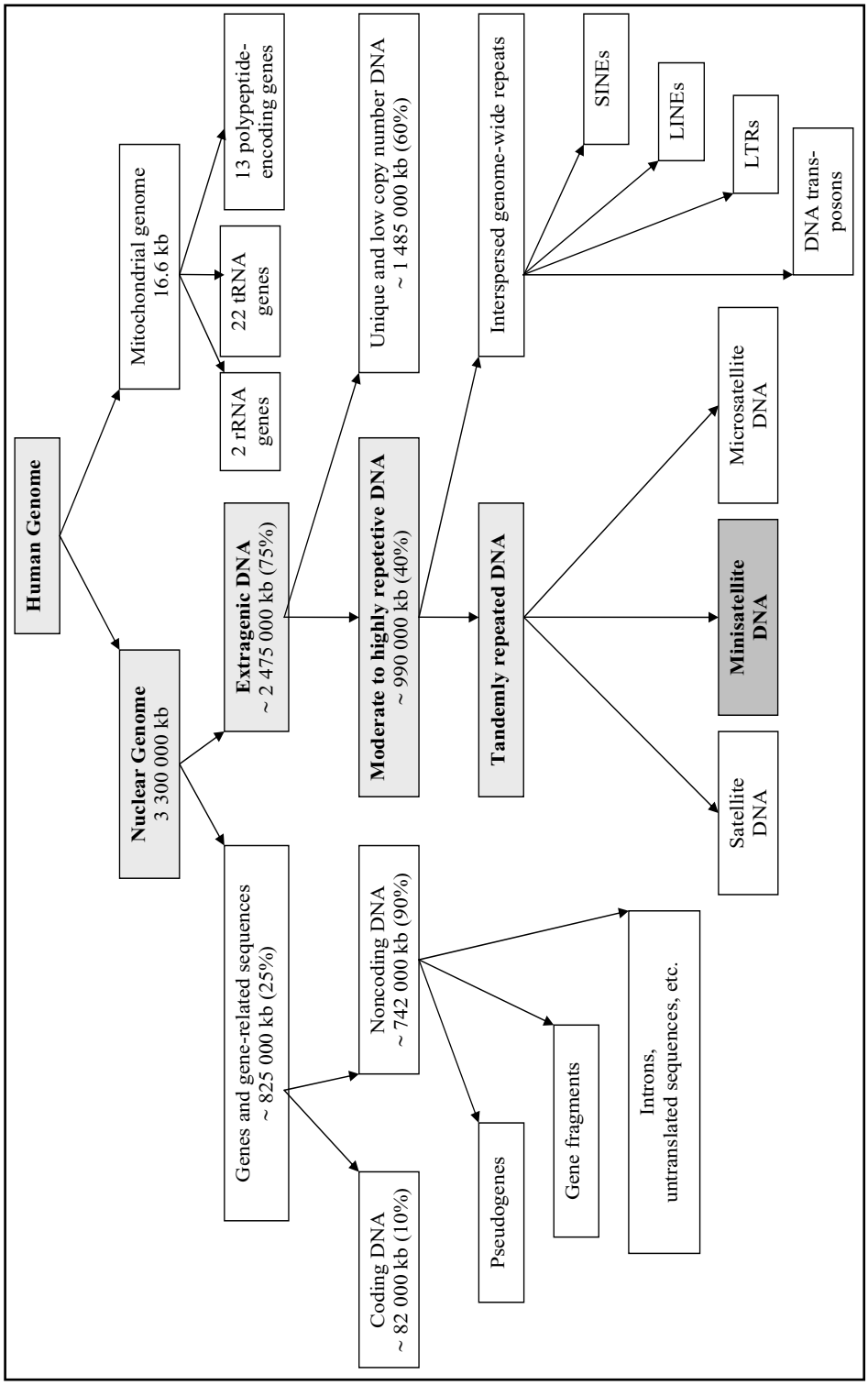

Figure 1. The organization of the human genome (modified from Strachan and Read 1999).

nucleotide called a cap is attached at the 5´end and a poly (A) tail is attached to the 3´end of the mRNA (end-modification). Chemical modification of mRNA is uncommon but it is seen in a diverse group of eukaryotes (RNA editing). After these processing events (splicing, end-modification, and RNA editing) the mRNA is transported to the cytoplasm for protein synthesis (translation) to occur on ribosomes (cellular components composed of proteins and another class of RNA). In translation, the mRNA is the direct molecular instruction for a specific protein's synthesis. Each specific sequence of three DNA bases (codons) forming the genetic code directs the cells protein-synthesizing machinery to add specific amino acids. Translation begins with an initiation codon, which is almost always AUG, specifying methionine (a few cases are known where ACG, CUG, or GUG are used instead) and ends with a termination codon (i.e., UAA, UAG, or UGA in the case of nuclear-encoded mRNA; UAA, UAG, AGA, or AGG in the case of mitochondrial-encoded mRNA). Ribosomes read the genetic code from the mRNA, and transfer RNAs (tRNAs) transport amino acids to the ribosomes for attachment to the growing protein. As with DNA and RNA, proteins are synthesized like 'beads on a string', but with 20 different kinds of beads (amino acids) rather than the four of DNA or RNA. In addition to 20 normal amino acids, two abnormal amino acids, selenocysteine and pyrrolysine, have been found. These are coded by codons (UGA and UAG), which are normally termination codons. The synthesized polypeptide is inactive until it is folded into its correct tertiary structure that defines its particular function and the activities that the cell is able to carry out. Selective gene readout in the form of RNA can govern the identity and quantity of proteins. The constellation of proteins in a cell is called its proteome. Proteins regulate cell replication, survival, and even death. Proteins work often by assembling into larger multiprotein complexes, which execute functions as protein synthesis and degradation, cell-to-cell signalling, and a host of other operations.

2.2 Polymorphic markers

The human genome is highly polymorphic and many different types of genetic variation can be observed in the DNA of modern humans. With the exception of identical twins, each person has a unique combination of genetic variants and will differ at many positions from the DNA sequence of the human genome resulting from The Human Genome Project. These individual variations in the DNA sequence can be studied by polymorphic markers. A polymorphism is a Mendelian or monogenic trait that exists in the population in at least two phenotypes (and presumably at least two genotypes), neither of which occurs with a frequency of less than 1-2%. In terms of DNA sequence, polymorphism exists in the human genome in a wide variety of forms. The most widespread is simple base substitution type of variation, but other types of variation, including tandem repeat variation, can be both extensive and functionally important. In the early 1980s, various polymorphic DNA markers provided, for the first time, a set of markers that were sufficiently numerous and spaced across the entire human genome to be used for searching variation in the human genome.

2.2.1 Minisatellites

Minisatellites are the first multiallelic polymorphic loci described in human DNA (Wyman and White 1980; Higgs et al. 1981; Bell et al. 1982). They form a new type of DNA markers based on the insertion or deletion of DNA. Minisatellites are characterised by successive blocks of oligonucleotides of variable lengths, thus this variation in the number of short, tandemly repeated sequences is also referred to as a variable number of tandem repeats (VNTRs) (Jeffreys et al. 1985; Nakamura et al. 1987; Wong et al. 1987a). Family studies have demonstrated that simple tandem repeat loci are inherited in a co-dominant Mendelian fashion (Kovacs et al. 1990).

2.2.1.1 Structure and localization of human minisatellites

Minisatellites are very commonly used polymorphic markers in the human genome, consisting of tandemly repeated DNA sequences. Minisatellites are generally GC-rich sequences characterized by a variable number of tandem repeats of identical 6-100 bp units. They are composed of repetitive highly variable ('hypervariable') sequences with a very large number of alleles. Minisatellites are highly informative because of this multiallelicity. Exceptionally high levels of polymorphic variation at these loci are due to variation in the number of tandem repeats. In addition to repeat number polymorphism (variation in array length), minisatellites also show core sequence polymorphism (variation in internal allelic structure) comprising different core variants and variation in the order in which

these variants are iterated in tandem. However, minisatellites share a common core sequence, GGGCAGGAXG (Jeffreys et al. 1985), which is similar in size and in G content to the *chi* sequence, a signal for generalized recombination in *E. coli*. Minisatellites can be grouped into sets according to their core sequence that is exclusive to each set and is shared by all members of that set. It has been suggested (Jeffreys et al. 1985) that core sequence may act as recombination signal in the generation of hypervariable regions (HVRs). Minisatellite alleles are rarely, if ever, sequence-homogeneous, but instead are composed of two or more variant repeat types present along the repeat array (Jeffreys et al. 1985; Wong et al. 1987a). The minisatellite repeat sequences are repeated from tens to thousands of times and thus the overall size of minisatellite clusters, in humans, can range from 0.5 to 100 kb. By using minisatellite variant repeat mapping by the PCR (MVR-PCR) method (Jeffreys et al. 1991a; Stead and Jeffreys 2002), the interspersion patterns of variant repeats within minisatellite alleles can be analysed by PCR amplification between a universal primer which anneals outside the repeat array, and primers which bind to specific variant repeats within the array. This method provides detailed internal structural information on an allele. MVR-PCR has been developed for several minisatellites and it has revealed that variant repeats are common and are almost always heavily intermingled with each other along an allele, implying that the germline mutation process must actively scramble the order of the repeat units. Given the high mutation rate of minisatellites, the true level of allelic diversity in humans is likely to be enormous, e.g., with $\geq 10^8$ different and distinguishable minisatellite alleles present at *MS32* locus in the world population (Jeffreys et al. 1991a).

Minisatellites are usually located in noncoding DNA, i.e., introns or untranslated regions (UTRs). However, there are few exceptional minisatellites that form a part of a gene (see 2.2.1.4.). There are altogether $>10^4$ potential minisatellite loci in the human genome. Minisatellites have been found to occur at over 2 000 highly polymorphic (hypervariable) loci dispersed throughout the genome with a relatively high frequency. Most (90%) of human minisatellites are found in subtelomeric regions (Royle et al. 1988). However, several minisatellite DNA sequences also occur at other chromosomal locations.

2.2.1.2 Mutation mechanisms and types at human minisatellites

In humans, instability at GC-rich minisatellites seems to involve different mutation processes operating in somatic and germline cells. Minisatellites are spontaneously unstable during germ cell development, although they are generally stable during somatic (blood) cell division. Low-frequency somatic minisatellite mutations are simple intra-allelic rearrangements; they consist usually of gains (duplications) or losses (deletions) of only few repeat units

located at random along the repeat array (Jeffreys et al. 1994; May et al. 1996; Jeffreys and Neumann 1997; Buard et al. 2000). The simple changes in new length alleles in blood could arise by mitotic recombination or unequal sister chromatide exchange; replication slippage does not appear to be a dominant process (Wolff et al. 1989; Jeffreys et al. 1997).

In sharp contrast to the simple minisatellite mutations in blood cells, sperm mutants can be very complex. Enhanced minisatellite mutation rate can result from damage accumulated in germ cells before meiosis, but does not necessarily indicate that the mutation events themselves occur pre-meiotically rather than later, for example at meiosis (Bois and Jeffreys 1999; Jeffreys et al. 1999; Tamaki et al. 1999; Buard et al. 2000; Stead and Jeffreys 2000). If so, then exposure to ionising radiation could potentially affect the stability of minisatellite loci over a very short interval of meiosis. New alleles at these highly unstable loci generally arise by germline-specific complex recombination events, including both interallelic gene conversions and intra-allelic rearrangements (Armour et al. 1993; Buard and Vergnaud 1994; Jeffreys et al. 1994, 1997, 1999; May et al. 1996; Jeffreys and Neumann 1997; Tamaki et al. 1999). Mutants are also generated via a crossover pathway, but at a lower frequency (Jeffreys et al. 1998a; Buard et al. 2000). Unequal crossing over due to misalignment of sister chromatids at mitosis or homologous chromosomes at meiosis leads to random increase by duplication or decrease by deletion of repeat units.

Most minisatellite loci show a bias toward minisatellite mutation in the male germline (Henke and Henke 1995; Dubrova et al. 1997). Sperm mutation is dominated by the inter-allelic transfer of blocks of repeat units from one allele (the donor) to the other (the recipient). While some transfers are simple, the majority are complex, with rearrangements including deletions or duplications in the recipient allele, scrambling of repeat units during transfer, and imperfect reduplications of donor-recipient junctions. In some cases, these rearrangements can be so extreme as to profoundly remodel allele structure in a single, astonishingly complex, sperm mutation event (Jeffreys et al. 1994). When individual sperm cells were studied, all studied minisatellite loci showed a bias towards gaining rather than loosing repeats (Jeffreys et al. 1994; May et al. 1996). Some minisatellite loci show polarity, with mutation events targeted to one end of the repeat array (Jeffreys et al. 1991a, 1994).

The polarity of the minisatellite mutations show that the minisatellite mutation process does not just reflect the instability of tandem repeat DNA, but it is actively controlled by elements external to the tandem repeats. It appears that minisatellites themselves are not so unstable; rather the instability is directed from a locally acting regulator. Flanking DNA contains elements necessary to initiate recombination and conversion and it basically switches minisatellite

allele on. A cis-acting regulator of minisatellite mutation is demanded for the allele to be activated for mutation. This initiator creates a double strand break (DSB) at the beginning of the repeat unit. The resulting gap is repaired using information from the other sister chromatid (or more often from the other allele). DSB or staggered nicks in the recipient allele are followed by pairing with the other allele and strand invasion and they produce a recombination complex. In most instances, and for unknown reasons, this complex is aborted to yield a conversion product. The abortion process must be complex to give the extraordinary rearrangements that can accompany gene conversion, which involves chunks of DNA being shifted from one allele to another during the mutation process. Occasionally, the recombination complex is correctly processed to yield a true meiotic recombinant. This model predicts the existence of a recombination/conversion initiator, adjacent to the repeat array, which controls tandem repeat instability and then variation in mutation rates across different loci could reflect differences in initiator efficiency. It has indeed been shown that there is a highly localized recombination hotspot upstream of the MS32 minisatellite extending into the beginning of the minisatellite (Jeffreys et al. 1998b). Also mutational process of CEB1 has been shown to be driven by the presence of meiotic recombination hotspot located just upstream of the repeat region and extending into the 5´region of the array (Buard et al. 2000). These recombination hotspots are responsible for driving repeat turnover at CEB1 and MS32 and thus minisatellites may evolve as by-products of localized meiotic recombination in the human genome.

The mutational processes among minisatellite loci are not completely solved and exceptional cases may be revealed by additional studies. One example is minisatellite MS1 which is an unusual minisatellite because it has a short repeat unit of 9 bp, it shows somatic instability in colorectal carcinomas (suggesting that replication or repair errors might contribute to MS1 repeat instability), and it has two distinct classes of structural change (Berg et al. 2003). These authors state that structural changes of the first class involves large and frequently complex rearrangements most likely arising by recombinational processes as seen at other minisatellites. However, structural changes of the second class generate exclusively single-repeat deletions restricted to sequence-homogeneous regions of alleles. The frequency of these changes depends on the length of uninterrupted repeats. The hyperinstability threshold seems to be similar in length to that observed at triplet-repeat loci showing expansions driven by dynamic mutation. The longest homogeneous repeats can have 0.7-1.3 deletions/sperm cell making these repeats extremely short-lived. These deletions can be generated by slippage during meiotic recombination or by post-meiotic repair. Post-meiotic repair has been seen in mice transgenic for Huntington disease CAG repeats, in which

expansion arises in post-meiotic haploid cells via repair of DNA breaks generated during sperm maturation (Kovtun and McMurray 2001). Berg et al. (2003) had no explanation for how these hyperdeleting repeats can come into existence and persist in human populations.

2.2.1.3 Spontaneous mutation frequencies of minisatellite loci

Minisatellite loci show high inter-allelic, inter-locus, inter-individual and inter-species variation in mutation rates. In humans, spontaneous minisatellite mutation frequencies are lower in somatic cells, i.e., between $2 \cdot 10^{-5}$ to $7 \cdot 10^{-5}$ (Jeffreys and Neumann 1997). However, in the germline, minisatellite mutation frequencies of the order of 10^{-1} to 10^{-3}/locus/ gamete/generation are much higher than the classical mutation rate of most protein-coding genes of 10^{-5} to 10^{-6}/locus/ gamete/generation (Jeffreys et al. 1985, 1988, 1991b, 1997; Kovacs et al. 1990). Spontaneous germline mutation frequencies vary not only between minisatellites but also between alleles at a given minisatellite locus (Monckton et al. 1994; May et al. 1996; Buard et al. 1998; Tamaki et al. 1999). For example, the mean mutation rate for individual alleles in men at the *MS205* minisatellite locus varies up to 20-fold (Zheng et al. 2000). Therefore, there is no general mutation rate for each minisatellite locus because the number of elements at any of these loci may vary from individual to individual and because the mutation rate depends upon the number of repeats. Minisatellite mutation shows a curious dependence on allele length; for minisatellites B6.7 and CEB1 minisatellite mutation rate increases steadily up to 40–50 repeats, above which it appears to reach a plateau (Jeffreys et al. 1997; Buard et al. 1998). The mutation rate of a newly arising allele differs from that of its parent. Germline mutation frequency variation has also been linked to a flanking polymorphism that appears to influence hotspot activity (Jeffreys et al. 1998b; Buard et al. 2000).

Minisatellites include some of the most variable loci in the human genome, and therefore minisatellite mutations are relatively easy to detect. The most dramatic variation is observed at *CEB1*, where sperm mutation rates vary from <0.05% to 25%/allele, the latter being the highest rate yet observed at a minisatellite (Buard et al. 1998). A minisatellite is classified as hypermutable if its average mutation rate in the germline is over 0.5%. Some 10% of human minisatellites are thus classifiable as hypermutable loci (Bois 2003). The observed high mutation rates are true both for human and mouse (expanded simple tandem repeats, ESTRs in mouse) minisatellite loci (e.g., Jeffreys et al. 1987, 1988, 1997; Kelly et al. 1989, 1991; Kovacs et al. 1990; Suzuki et al. 1993; McKusick 1998). These higher mutation rates lead to a much higher level of polymorphism between unrelated individuals within a population. At the same time, the mutation frequency is low enough for minisatellite alleles to be followed easily in classical breeding studies.

2.2.1.4 Biological function of human minisatellites

The great majority of hypervariable minisatellite DNA sequences are not transcribed, except for elements occurring within noncoding intragenic sequences. However, there are a few exceptional cases in the human genome, and a fair number of cases outside the human genome, of minisatellites that actually form part of genes. There are tandem repeated DNA sequences [e.g., mucin (*MUC1*) locus on 1q] that code for tandem repeated protein sequences. *MUC1* locus encodes a glycoprotein found in several epithelial tissues and body fluids. It is highly polymorphic as a result of extensive variation in the number of minisatellite-encoded repeats (Swallow et al. 1987a, b; Lancaster et al. 1990). Another example of coding minisatellites is human apolipoprotein family. Each member of this family contains a coding minisatellite with a repeat unit of 33 or 66 bp (Boguski et al. 1986). Apolipoproteins are capable of binding lipids, each being associated with a lipoprotein of specific density (Mahley et al. 1984). This specificity is due to the variable coding minisatellite region in each gene. In addition, involucrin, loricrin and small proline-rich (SPR) genes (Eckert and Green 1986; Gibbs et al. 1993a) as well as the D4 dopamine receptor (D4DR) (Van Tol et al. 1992; Lichter et al. 1993) contain coding minisatellites. The coding polymorphisms observed in *D4DR* gene have been shown to affect its ligand-binding affinity (Van Tol et al. 1992; Asghari et al. 1994). It is also associated with cognitive and emotional disorders as a consequence of the high level of D4DR expression in the limbic areas of the brain (Benjamin et al. 1996, 1997; Ebstein et al. 1996, 1997). In addition, the prion protein gene contains a 24 bp repeat region coding for an octapeptide. Some inherited forms of prion diseases, including Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler Scheinker syndrome (GSS) are associated with the amplification of 4–9 extra repeats in the coding repeated region (Palmer and Collinge 1993). The biological consequences of these amplifications remain unclear. Finally, minisatellite CEB205 appears to contribute to the coding sequence itself (Denoued et al. 2003).

Hypervariable minisatellite DNA has been reported as a hotspot for homologous recombination in human cells (Wahls et al. 1990). Minisatellites may act as 'harmless' places for homologous recombination, thus minimizing the effects of possible errors (mutations) in this process. The minisatellite mutation process, controlled by some elements near the minisatellite, may reflect some basic biological process going on in DNA. Although most minisatellite mutations are likely to be without phenotypic consequence, there are some exceptions where the minisatellites have been implicated as regulators of gene expression (for example, at levels of transcription, alternative splicing, or imprint control) or as a part of bona fide open reading frames (see 2.2.1.5). Minisatellites have also been associated with chromosome fragile sites and are proximal to a number of recurrent translocation breakpoints (Sutherland et al. 1998).

2.2.1.5 Possible mechanisms of repeat diseases

Tandem mini- and microsatellite (see 2.2.2) repeats can cause different human diseases (Table 1) by several possible mechanisms. The best-studied examples are diseases caused by an expanded trinucleotide repeat. Diseases caused by expansion of an unstable CAG repeat within a gene are all late-onset neurodegenerative diseases and except for spinal and bulbar muscular atrophy (Kennedy disease, On-Line Mendelian Inheritance in Man = OMIM 313 200), are all dominantly inherited. No other mutation in the gene has been found to cause the disease. The expanded allele is transcribed and translated and encodes a protein of unknown function. The CAG repeat encodes a polyglutamine tract in the protein. There is a critical threshold repeat size, below which the repeat is non-pathogenic and above which it causes disease. When the polyglutamine tract exceeds the threshold length the protein aggregates, forming an inclusion body, which apparently kills the cell (Kim and Tanzi 1998). The larger the repeat, above the threshold, the earlier is the age of onset (on average, predictions cannot be made for individual patients, but there is a clear statistical correlation). The androgen receptor mutation in Kennedy disease provides clear evidence that CAG-repeat diseases involve a specific gain-of-function. Loss-of-function mutations in this gene are well known and cause androgen insensitivity or testicular feminization syndrome (OMIM 300068), a failure of male sexual differentiation. The different clinical features of each disease reflect the killing of different cells, presumably because of interactions with other cell-specific proteins. The mechanisms and their general significance remain to be discovered.

Minisatellites have also been implicated as regulators of gene expression (for example, at levels of transcription, alternative splicing, or imprint control) or as part of bona fide open reading frames (reviewed by Bois and Jeffreys 1999). In Fragile-X syndrome and Friedreich's ataxia (Table 1), an enormously expanded repeat causes a loss-of-function by abolishing transcription. The same is true for the expanding 12-, 15-, or 18-mer in juvenile myoclonus epilepsy. In each case, the disease is occasionally caused by different, more conventional, loss-of-function mutations in the gene. Such mutations produce an identical clinical phenotype as expansions, apart from (presumably) not showing anticipation.

The minisatellite repeats have also been envisaged as possible protein-binding sites and in some cases, protein binding at the RNA level has also been suggested to contribute to pathogenesis, notably in dystrophia myotonica (Philips et al. 1998). No other mutations have ever been found in dystrophia myotonica patients, so there must be something quite specific about the action of the CTG repeat. It may affect processing (splicing) of the primary transcript in a specific way, or it may affect expression of a whole series of genes by altering the chromatin structure in this gene-rich chromosomal region. The site of the

expansion reduces expression of the adjacent gene. SCA8 may have a similar mechanism (Koob et al. 1999).

The non-random clustering of chromosome deletion breakpoints with the CCG repeats has given reason to assume that they may play an important role in a common mechanism of chromosome breakage. This has been shown, e.g., in the fragile 11B site causing the Jacobsen (11q-) syndrome (Jones et al. 2000). Other highly expanded shorter repeats, such as FRA16A (an expanded CCG repeat) (Nancarrow et al. 1994, 1995) or FRA16B (an expanded 33 or 35 bp minisatellite) (Yu et al. 1997; Yamauchi et al. 2000) are non-pathogenic, presumably because no important gene is located nearby.

There is evidence that certain minisatellite mutations can regulate or modify the transcription of adjacent genes and therefore they might be at the origin of the associated pathologies. This has been shown to occur with the insulin gene, the cystatin B gene and *HRAS* oncogene. The insulin minisatellite (INS VNTR OMIM 147510) located 596 bp upstream of the human insulin gene translation-initiation site, has attracted widespread interest because of its associations with a range of phenotypes, including susceptibility to type 1 diabetes (Bennett et al. 1995; Bennett and Todd 1996), insulin dependent diabetes mellitus type 2 (IDDM2) (Bell et al. 1984), polycystic ovary syndrome (Wa-terworth et al. 1997), variation in birth size (Dunger et al. 1998), and obesity (Le Stunff et al. 2001). In IDDM2, repeat alleles of different length have been shown to affect transcriptional regulation of insulin (*INS*) gene expression, with short alleles resulting mainly in increased insulin expression and susceptibility to IDDM2 (Bell et al. 1984; Bennett et al. 1995; Kennedy et al. 1995; Lucassen et al. 1995). In the case of the cystatin B, the expansion of the dodecamer repeat located in the proximal promoter of the cystatin B gene severely disrupts the function of the promoter and thus reduces transcription (Pennacchio et al. 1996; Lalioti et al. 1997; Virtaneva et al. 1997; Alakurtti et al. 2000). *HRAS* oncogene associated minisatellites have shown to act as risk factors for spontaneous abortion (Kiaris et al. 1995) and various cancers (Trepicchio and Krontiris 1992; Green and Krontiris 1993; Krontiris et al. 1993; Phelan et al. 1996; Gosse-Brun et al. 1999; Lindstedt et al. 1999; Rosell et al. 1999; Weitzel et al. 2000). However, the link between the HRAS minisatellite and cancer remains uncertain as in many studies the cancer association could not be seen (Thein et al. 1986; Sutherland et al. 1986; Ishikawa et al. 1987; Gerhard et al. 1987; Firgaira et al. 1999). Langdon and Armour (2003) explained this discrepancy by the possibility that earlier studies showing cancer association could have been incapable of distinguishing small length differences at the HRAS minisatellite because these studies were conducted using Southern blot hybridization compared with more recent PCR-based studies.

2.2.1.6 Exploitation of minisatellite polymorphisms

Minisatellites were originally utilized as highly informative hypervariable markers for linkage analysis (Lander and Schork 1994), but rapidly swept to prominence with the realization that they provided a powerful new method for individual identification. Thus a major application of minisatellites has been in DNA fingerprinting. Minisatellite DNA fingerprinting has been exploited in paternity and immigration disputes, forensic studies, zygosity testing, and monitoring bone marrow transplants. Minisatellites are efficient markers for gene mapping and loss of heterotsygosity (LOH) studies. Single locus probes are used in pedigree analysis and mutation screening, where significant changes can be detected with smaller sample sizes. Minisatellites are nowadays widely used also in identification of different strains of bacteria and in molecular epidemiology. When mycobacterial interspersed repetitive units (MIRUs) in 12 human minisatellite-like regions were studied in Mycobacterium tuberculosis genome, $>16 \cdot 10^6$ different combinations were found. This has been utilized in MIRU-VNTR identification of tuberculosis (Mazars et al. 2001).

2.2.2 Microsatellites

In addition to minisatellites, the other type of multiallelic polymorphic loci in the human genome consists of microsatellite markers (Litt and Luty 1989; Weber and May 1989) or short tandem repeats (STRs). Microsatellites are composed of 1–4 bp repeating units. Repeat units of (AT)n are the most common ones in humans and (AC)n in rats (Beckman and Weber 1992). Microsatellite cluster length is usually <150 bp, but some STRs involved in diseases have thousands of repeats. Microsatellite markers account for about 0.5% of the human genome and they occur relatively evenly spaced throughout the genome approximately once in every 30 kb (Strachan and Read 1999). Thus, the potential number of microsatellite loci in the human genome is $>10^5$, out of which about 10^4 microsatellites have already been discovered. Microsatellites are spread throughout the human genome and are located mostly in introns and untranslated regions. Many of them are polymorphic (i.e., variable from individual to individual) in copy number in human populations due to the high spontaneous mutation rates estimated to be in the range of 10^{-2} to 10^{-4}/locus/gamete/generation (Ellegren 2000). Microsatellite mutations are 5–6 times more frequent in the human male germline than in the female germline (Brinkmann et al. 1998). Large-scale pedigree studies have shown that more than 90% of microsatellite mutations result from the expansion or contraction of alleles by a single repeat unit. Repeat length and base composition affect the microsatellite mutation rate; dinucleotide repeats mutate faster than trinucleotide repeats

and sequences with high AT content mutate faster than sequences with high CG content. In addition, the number of repeats, the presence of interruptions within the sequence, and the rate of intracellular processes such as replication, transcription, DNA repair, and recombination may contribute to the mutational dynamics of microsatellite DNA (Schlötterer 2000). Experiments in bacteria, yeast, and mammalian systems suggest that an elevated frequency of length changes (expansions and deletions) is caused by their propensity to form unusual secondary structures (McMurray 1999). Unstable DNA structures such as left-handed Z-DNA, cruciforms, slipped-stranded DNA, triplexes, and tetraplexes may form due to their palindromic nature, under physiological conditions as well as *in vivo* (Jaworski et al. 1987; Sen and Gilbert 1988; Parniewski et al. 1990; Pearson and Sinden 1996). Such structures may be potentially hazardous for genome stability if not removed by repair mechanisms.

Microsatellite mutations are selectively neutral and so they are compatible with the assumptions of most population genetic theory. There are many microsatellites with 5–10 different length alleles, and a few with even more. Therefore, they are highly informative and provide a rich source of markers that are widely used in studies of the human genome. PCR amplification of microsatellite markers is utilized in personal identification (e.g., forensic and paternal analysis), quantitative analysis of chimerism after allogenic stem cell transplantation (recently reviewed by Acquaviva et al. 2003), genetic mapping, and linkage analysis in association with disease susceptibility genes. One form of microsatellites, unstable trinucleotide repeats, in 5' and 3' untranslated regions and coding regions are known to be associated with several inherited neurological disorders (Ashley and Warren 1995; Warren 1996). Altogether, 20 Mendelian diseases have been shown to be associated with trinucleotide repeat expansions (Table 1). In general, there is an inverse relationship between the age of onset and the size of repeat and a direct relationship between expansion size and disease severity. The length of the expanded trinucleotide repeat sequence in afflicted families may vary from as much as tens of triplet repeats to thousands of repeats.

2.2.3 Single nucleotide polymorphism

Apart from micro-and minisatellites, all other types of human polymorphisms are mostly biallelic. The most widespread class of variation in the human genome is single nucleotide polymorphism (SNP) (Kruglyak 1997; Wang et al. 1998). SNPs are simple base-substitutional variants in DNA sequence that comprise a large part of human diversity. They are very frequent, occurring once per 1250 bp on average but there is marked heterogeneity in the level of SNP polymorphism

across the human genome (Venter et al. 2001). More than 5 million SNPs with a minor-allele frequency greater than 10% are expected to exist in the human genome (Kruglyak and Nickerson 2001). A recent analysis (Reich et al. 2003) indicated that over half (2.7 million) of SNPs with frequency of >10% are already presented in available databases and thus can be utilized. The public (TSC and BAC overlap) and Celera databases contain approximately 4 million candidate SNPs in the human genome (Sachidanandam et al. 2001; Marth et al. 2001; Venter et al. 2001). However, at present the databases contain limited information about the population frequency of SNPs. The frequency of base-substitutional polymorphism depends on local sequence context. The dinucleotide CG, in which the cytosine is generally methylated in human DNA, has a relatively high mutation rate to TG or CA. As a result, CG dinucleotides are preferential sites of polymorphism as well as of disease-causing mutations in genes. As each SNP has usually only two alleles, SNP typing is easily automated and widely used in mapping disease associations. Less than 1% of all SNPs result in variation in proteins, but the task of determining which SNPs have functional consequences is an open challenge. Studies have revealed SNP variation in and around genes of medical importance and it has been suggested that their inheritance may alter susceptibility to diseases (Lander 1996; Risch and Merikangas 1996; Collins et al. 1997). SNPs may have effects (e.g., individual susceptibility to cancer) that are not recognised, but which may be yet important in future human genetics.

2.3. Mutations in the human genome

In a living cell, DNA undergoes frequent chemical change, especially when it is replicated (in S phase of the eukaryotic cell cycle). The DNA of the human genome is subject to a variety of different types of hereditary change (mutation). A mutation is a change in the nucleotide sequence of a genome. Mutations result either from errors in DNA replication and repair (spontaneous or endogenous mutations) or from the damaging effects of mutagens such as radiation and chemicals that react with DNA (induced or exogenous mutations). Most DNA damage in humans is spontaneous and caused by thermodynamic decay processes and by reactive free radicals formed by the oxygen metabolism. Each mammalian cell suffers millions of spontaneous DNA-damaging events/year. Thus, all cells have effective DNA repair systems that attempt to identify and correct abnormalities in the DNA sequence in order to minimise the number of mutations. The ultimate source of all variation in the human genome is *de novo* mutation in the germline, resulting in sequence alterations that can range from simple base substitutions through deletion, duplication, and transposition to turnover at tandem repeat loci.

2.3.1. Molecular nature of mutations

The clinical effects of various changes in a given gene can be observed in humans. Many different mutations are known for most inherited diseases where the responsible gene has already been identified. On September 9, 2004, The Human Gene Mutation Database (HGMD) (http://archive.uwcm.ac.uk/uwcm/mg/docs/hahaha.html) contained 42 521 mutations in 1 657 human genes (Table 2). Currently 1 364 different mutations have been described in the cystic fibrosis (CF) (OMIM 219 700) gene (http://www.genet.sickkids.on.ca/cftr) and 390 in the haemoglobin beta (HBB) (OMIM 141 900) gene (http://archive.uwcm.ac.uk/uwcm/mg/hgmd/search/119297.html).

A comparatively common type of human mutations consists of point mutations (single-base substitutions), which replace one nucleotide with another. Point mutations include transitions and transversions. In a transition, one purine is substituted by another purine (A↔G) or one pyrimidine is substituted by another pyrimidine (C↔T). In a transversion, a purine is replaced by a pyrimidine (A→T, A→C, G→T, or G→C) or a pyrimidine is replaced by a purine (C→A, C→G, T→A, or T→G). Missense mutations alter the codon so that it specifies a different amino acid. Often this has no significant effect on the biological activity of the protein. In nonsense mutations, a codon for an amino acid is changed into a terminating (STOP) codon (UAA, UAG, or UGA in nuclear mRNA; UAA, UAG, AGA, or AGG in mitochondrial mRNA). Therefore, translation of the mRNA transcribed from this mutant gene will stop prematurely, resulting in a shortened or absent protein. However, nonsense mutations often trigger mRNA instability rather than cause production of a truncated protein. Missense/nonsense mutations constitute 57% of the over 42 521 human mutations that have been collected in the Human Gene Mutation Database (HGMD) (Table 2). Silent substitutions result in a new codon specifying the same amino acid. They usually cause no change in their product and can be detected only by sequencing the gene (or it is mRNA). Silent mutations also include virtually all those that occur in extragenic DNA and in the noncoding components of genes and generelated sequences (Figure 1). Consequently, 98.9% of the human genome can be usually mutated without significant effect. Mutations in the coding regions of the genes are much more important. Multiple base substitutions (gene conversion-like events) are rare except at certain tandemly repeated loci or clustered repeats. Base substitutions in coding sequences may be pathogenic because splice site mutations create or destroy signals for exon-intron splicing. They may also destroy an embedded signal (e.g., a nuclear localization signal).

In addition to point mutations and multiple base substitutions, human mutations also contain insertions, duplications, and deletions. In insertions, one or more nucleotides are inserted into a DNA sequence. Insertions of one or a few

Table 1. Human diseases known to be caused by micro- or minisatellite tandem repeats.

Disease	OMIM number[25]	Mode of inheritance	Location of the gene	Repeat sequence	Location of repeat	Repeat number	
						Normal	Disease
***DM*[1]**	160 900	AD[26]	19q13.3	CTG	3'UTR[30]	5-37	50-4 000
***DM2*[2]**	602 668	AD	3q13.3-q24	CCTG	intron 1	75-	-11 000
***DRPLA*[3]**	125 370	AD	12p13.31	CAG	coding	3-35	49-93
***EPM1*[4]**	254 800	AR[27]	21q22.3	CCCCG-CCCCGCG	promoter	2-3	30-80
***FRAXA*[5]**	309 550	X[28]	Xq27.3	CGG	5'UTR	5-54	200->1 000
***FRAXE*[6]**	309 548	X	Xq28	CCG	promoter	6-25	200-750
FRA 11B/ Jacobsen	600 651	mf[29]	11q23.3	CGG	5' UTR	11	100-1 000
***FRDA*[8]**	229 300	AR	9q13, 9p23-p11	GAA	intron 1	7-22	120-1 700
***HD*[9]**	143 100	AD	4p16.3	CAG	coding	6-35	36-121
***HRS*[10]**	140 340	AD	12p13.31	CAG	coding	3-13	63-68
***IDDM2*[11]**	125 852	mf	11p15.5	ACAGGGG-TGTGGGG	5'UTR	138-210	26-63
***OPMD*[12]**	164 300	AD	14q11.2-q13	GCG	coding	6	8-13
***OPMD*[13]**	257 950	AR	14q11.2-q13	GCG	coding	6	7

[1] Dystrophia myotonica. One form of dystrophia myotonica has been found to be caused by a CTG expansion near the dystrophia myotonica protein kinase gene (*DMPK*, OMIM 605377) (Brook et al. 1992; Mahadevan et al. 1992).
[2] Dystrophia myotonica 2 is caused by expansion of a CCTG repeat in intron 1 of zinc finger protein9 (*ZNF9*, OMIM 116955) (Liquori et al. 2001).
[3] Dentatorubral-pallidoluysian atrophy is caused by the same expanded CAG repeat as HRS (Burke et al. 1994; Koide et al. 1994; Ikeuchi et al. 1995; Takiyama et al. 1999; Shimojo et al. 2001).
[4] Progressive myoclonus epilepsy is caused by 12 bp expansions in the promoter region of the cystatin B gene (*CSTB*, OMIM 601145). In addition, 15bp and 18 bp repeated units have been shown to cause EPM1 (Pennacchio et al. 1996; Lalioti et al. 1997, 1998; Virtaneva et al. 1997).
[5] Fragile-X site (Oberlé et al. 1991; Verkerk et al. 1991; Yu et al. 1991; Bates and Lehrach 1994).
[6] Fragile-X site E (Knight et al. 1994).
[7] Fragile site 11B locates 5' prime end of the *CBL2* oncogene (OMIM 165360) which includes a CCG trinucleotide repeat. Fragile site 11B causes predisposition toward Jacobsen syndrome which is a chromosomal aberration (11q-), and many genes are involved in the phenotype of the offspring (Jones et al. 1995, 2000).
[8] Friedreich's ataxia. One form of Friedreich's ataxia is caused by mutation in the *FRDA* gene (OMIM 606829), which has been mapped to 9q. Another locus for the disorder has been mapped to 9p (OMIM 601992) (Campuzano et al. 1996; Dürr et al. 1996a; Filla et al. 1996; Delatycki et al. 1999, 2000).
[9] Huntington's disease (MacDonald et al. 1993; Ambrose et al. 1994; Nasir et al.1996).
[10] Haw River syndrome is caused by the same expanded CAG repeat as DRPLA (Burke et al. 1994).
[11] Insulin-dependent diabetes mellitus type 2 is caused by minisatellite expansion (INS VNTR, OMIM 147510) located 596 bp upstream of the insulin gene (*INS*, OMIM 176730) translation initiation site (Bell et al. 1982, 1984; Owerbach and Gabbay 1993; Bennett et al. 1995; Kennedy et al. 1995; Lucassen et al. 1995; Lew et al. 2000).
[12, 13] Oculopharyngeal muscular dystrophy is caused by mutation in the gene encoding poly(A) – binding protein-2 (*PABP2*, OMIM 602279) (Fried et al. 1975; Brais et al. 1998; Becher et al. 2001). Mode of the inheritance of the disease depends on the length of the GCG repeat.

Table 1. continues.

Disease	OMIM-number[25]	Mode of inherit-ance	Location of the gene	Repeat sequence	Location of repeat	Repeat number	
						Normal	Disease
SBMA[14]	313 200	XR	Xq11-q12	CAG	coding	6-35	36-72
SCA1[15]	164 400	AD	6p23	CAG	coding	6-44	39-83
SCA2[16]	183 090	AD	12q24	CAG	coding	13-33	32-77
SCA3/ MJD[17]	109 150	AD	14q32.1	CAG	coding	12-47	54-89
SCA6[18]	183 086	AD	19p13	CAG	coding	4-18	20-33
SCA7[19]	164 500	AD	3p12-p13	CAG	coding	4-35	36-306
SCA8[20]	603 680	AD	13q21	CTG	3'UTR	14-92	100-250
SCA10[21]	603 516	AD	22q13 - qter	ATTCT	intron 9	10-28	29-4500
SCA12[22]	604 326	AD	5q31-q33	CAG	5'UTR	7-45	55-78
SCA17[23]	607 136	AD	6q27	CAG	coding	25-42	44-63
SPD[24]	186 000	AD	2q31	GCG	coding	15	22-29

[14]Spinal and bulbar muscular atrophy (Kennedy disease) is caused by mutation in one part of the androgen receptor gene (*AR*, OMIM 313700) (Doyu et al. 1992; La Spada et al. 1992; Amato et al. 1993; Ferlini et al. 1995).
[15]Spinocerebellar ataxia type 1 (Orr et al. 1993; Banfi et al. 1994; Schöls et al. 1995).
[16]Spinocerebellar ataxia type 2 is caused by an expansion of a CAG repeat in the coding region of 1 isoform of the alpha-1A calcium channel subunit (*CACNA1A*, OMIM 601011) (Pulst et al. 1993, 1996; Imbert et al. 1996; Sanpei et al. 1996; Riess et al. 1997).
[17]Spinocerebellar ataxia type 3/Machado-Joseph disease (Kawaguchi et al. 1994; Giunti et al. 1995; Maruyama et al.1995; Schöls et al. 1995; Twist et al. 1995; Dürr et al. 1996b).
[18]Spinocerebellar ataxia type 6 is associated with CAG expansion in the α_{1A}-voltage-dependent calcium channel gene (*CACNL1A4,* OMIM 601011) (Matsuyama et al. 1997; Zhuchenko et al. 1997; Takiyama et al. 1998; Ishikawa et al. 1999; Mariotti et al. 2001).
[19]Spinocerebellar ataxia type 7 (David et al. 1997; Gouw et al.1998; Stevanin et al. 1998; Giunti et al. 1999; Michalik et al. 1999; Jonasson et al. 2000).
[20]Spinocerebellar ataxia type 8 has been shown to be caused by a CTG expansion (Koob et al. 1999; Moseley et al. 2000; Silveira et al. 2000; Stevanin et al. 2000; Vincent et al. 2000; Cellini et al. 2001; Topisirovic et al. 2002), but this expansion has also been shown not to be confined to patients with SCA8 (Worth et al. 2000; Sobrido et al. 2001).
[21]Spinocerebellar ataxia type 10 (Matsuura et al. 2000, 2002; Grewal et al. 2002; Potaman et al. 2003).
[22]Spinocerebellar ataxia type 12 is caused by an expansion of a CAG repeat in a brain-specific regulatory subunit of the protein phosphatase PP2A (*PPP2R2B*, OMIM 604325) (Holmes et al. 1999; Cholfin et al. 2001; Fujigasaki et al. 2001; Srivastava et al. 2001).
[23] Spinocerebellar ataxia type 17 is caused by an expansion of a CAG repeat in a TATA box-binding protein (TBP, OMIM 600075) (Koide et al. 1999; Nakamura et al. 2001; Zühlke et al 2001; Silveira et al. 2002).
[24]Synpolydactyly phenotype is caused by mutation in the homeo box D13 gene (*HOXD13*, OMIM 142989). However, this does not seem to be an unstable expanding repeat. The expansion is probably the result of unequal crossing over, and at least in one family it has been stable for 7 generations (Akarsu et al. 1996; Kjaer et al. 2002).
[25]On-Line Mendelian Inheritance in Man (OMIM) (McKusick 2001); http://www3.ncbi.nlm.nih.gov/Omim/searchomim.html
[26]Autosomal dominant mode of inheritance.
[27]Autosomal recessive mode of inheritance.
[28]X-chromosomal mode of inheritance.
[29]Multifactorial disease.
[30]Untranslated region.

nucleotides are very common in noncoding DNA but rare in coding DNA, where they produce frameshift (see below). Occasionally large-scale tandem duplications and insertions of transposable elements are found. In deletions one or more nucleotides are eliminated from the DNA sequence. The deletion size varies from 1 bp to megabases. Deletions of one or a few nucleotides are very common in noncoding DNA but are rare in coding DNA, where they produce frameshifts. Larger deletions are rare, but often occur in regions containing tandem repeats or between interspersed repeats. Insertions and deletions are particularly prevalent in short repeated sequences (e.g., microsatellites) because such sequences can induce replication slippage. In replication slippage, the template strand and its copy shift their relative positions so that part of the template is either copied twice or missed out. The result is that the new polynucleotide has a larger or smaller number of the repeat units. Microsatellite sequences are hypervariable, as replication slippage occasionally generates a new length variant and adds it to the collection of alleles already present in the population (see below: dynamic mutations). Insertions, deletions, or splicing errors result in frameshift mutations. If the number of bases inserted or deleted is not a multiple of three base pairs, the reading frame is changed (frame shift mutation) since the genetic code is read in non-overlapping triplets. This leads to the situation where the entire amino acid sequence of the protein is altered beyond the site of the mutation, causing loss-of-function of the protein.

A further class of human mutations contains large-scale chromosome abnormalities that involve loss or gain of chromosomes or the breakage and rejoining of chromatids. Numerical and structural chromosomal abnormalities are rarer as constitutional mutations because they are often lethal and end up in spontaneous abortion. They are far more common as somatic mutations and are often found in tumour cells.

The most recently found class of human mutations (dynamic mutations) arise as a result of non-traditional mechanisms. Dynamic mutations are unlike conventional mutations, because the expanded repeats can undergo further change (usually continued expansion) with each subsequent generation. For those expansions that result in an altered phenotype, the subsequent expansion of the repeat in descending generations can lead to non-Mendelian inheritance patterns due to incomplete penetrance and/or variable expressivity, which can generally be directly correlated with the parental repeat length. With the discovery that one form of microsatellites, an expanded CGG repeat, is the mutation causing Fragile-X mental retardation (Oberle et al. 1991; Yu et al. 1991), trinucleotide repeat expansions were recognised as an important and novel mutation mechanism in human genetic disease. Trinucleotide repeat expansions are rare but they can change tandem repeat sequences in coding or untranslated regions of genes and cause various human diseases (Table 1).

Table 2. Number of entries by mutation type in Human Gene Mutation Database (HGMD) statistics (September 9, 2004) (http://archive.uwcm.ac.uk/uwcm/mg/docs/hahaha.html).

Mutation type		Number
Micro-lesions		
	Missense/nonsense	24 364
	Splicing	4 055
	Regulatory	449
	Small deletions	7 089
	Small insertions	2 810
	Small indels[1]	400
Gross lesions		
	Repeat variations	88
	Gross insertions and duplications	390
	Complex rearrangements (including inversions)	500
	Gross deletions	2 376
Total		***42 521***

[1] Small insertion or deletion.

These different kinds of human mutations can be detected in various parts of the human genome. In addition to coding sequences, mutations have also been found in regulatory regions, promoter regions, splice junctions, within introns, and in polyadenylation sites. They may interfere with any stage in the pathway of expression, from the gene to the protein product. Point mutations causing a defect in mRNA splicing can cause a reduction in the amount of mRNA generated, production of an aberrant mRNA, and non-recognition by the cellular machinery of an exon or exons following the lesion. The non-recognised exons are excluded from the mature RNA transcript, a process called exon skipping. Most mutations, including all silent ones as well as many in coding regions, will still not change the phenotype of the organism in any significant way. Those having an effect can be divided into loss-of-function and gain-of-function mutations. Usually recessive loss-of-function mutations reduce or abolish a protein activity. One example of an exceptional dominant loss-of-function mutation is haploinsufficiency, where the organism is unable to fully tolerate the approximately 50% reduction in protein activity suffered by the heterotzygote (e.g., Marfan syndrome in humans). Proteins that require all homomers to be intact are another example of dominant loss-of-function mutations (e.g., ALDH2 functions as a homotetramer, and in heterozygotes only 1/16 of the availabe combinations are fully functional). Gain-of-function mutations confer an abnormal activity of a protein and are usually

dominant. Many gain-of-function mutations are in regulatory sequences rather than in coding regions. Therefore, they can have a number of consequences. For example, one or more genes can be expressed in wrong tissues, whereby these tissues gain functions that they normally lack. In other situations one or more cell cycle control genes are over expressed, thus leading to uncontrolled cell division and hence to cancer.

The above-mentioned different types of human mutations (except dynamic mutations) arise usually as new mutations in single individuals in somatic cells or in the germline. For the estimation of hereditary risks of radiation, the most important mutations are germline mutations as the individuals who inherit these mutations have them present in every cell of the body and they can be transmitted to the next generation.

2.3.2 Molecular techniques used for screening unknown mutations

As shown above, various types of human mutations can arise spontaneously or they may be induced by, e.g., radiation or chemicals. The great majority of mutations are not associated with disease but they occur within noncoding DNA sequences. During the last decades various molecular techniques have been developed for screening unknown mutations.

Traditionally used Southern blot hybridization (Southern 1975) uses restriction enzyme digestion of studied DNA samples. Digested DNA samples are fractionated by gel electrophoresis, blotted and hybridised to probes derived from the polymorphic locus. Studied polymorphic loci may be restriction fragment length polymorphisms (RFLPs) (Botstein et al. 1980) or a variable number of tandem repeats (VNTRs) (Jeffreys et al. 1985; Nakamura et al. 1987; Wong et al. 1987a). Unlike traditional point mutation RFLPs, minisatellites are caused by (and reflect) changes in the actual size of the locus itself. The analysis of VNTRs at a single-locus site results in the detection of two DNA fragments differing in length as long as the paternal and maternal alleles are not identical by coincidence. As the Southern hybridization method contains gel electrophoresis, it is laborious, relatively slow, and expensive. A further disadvantage of this method is the demand for quite large samples because it requires several µg of DNA. Conventionally run agarose gels will resolve fragments 500 bp–20 kb in size and therefore Southern blotting can only detect rearrangements in this size range. Rearrangements outside the size range detected by Southern blotting can be analysed using pulsed field gel electrophoresis (PFGE) (Collier et al. 1989). PFGE involves the restriction digestion of genomic DNA using rare-cutting enzymes, resulting in large restriction fragments. The extraction and digestion of the DNA is done in agarose blocks, which are then loaded directly

into the wells of the gel. Large restriction fragments are separated with a specialised electrophoresis system in which the direction of the electric field is changed/pulsed regularly. As in Southern blotting, rearrangements are detected as abnormally sized fragments upon hybridisation with a probe corresponding to the gene of interest. The RNA equivalent of Southern blotting is called Northern blotting. If the gene rearrangement significantly alters the length of the RNA transcript, it should be detectable by Northern blotting. Northern and Southern blotting can detect only a very small proportion of unknown mutations but they are useful as a preliminary mutation screen and they are the only way to detect large deletions, insertions, and duplications.

Formerly Southern blot–based mutation screening was revolutionised by the invention of the polymerase chain reaction (PCR) (Mullis et al. 1986; Saiki et al. 1988). PCR is usually designed to selectively amplify a specific target DNA sequence(s) within a heterogeneous collection of DNA sequences (e.g., total genomic DNA or a complex cDNA population). PCR is a rapid and simple method requiring minute amounts of DNA or cDNA and it is most suitable for sequences less than 300 bp in length. PCR is ideal for providing numerous DNA templates for mutation screening. Nowadays, minisatellites can also be typed by PCR less laboriously than by using Southern hybridization. The small pool polymerase chain reaction (SP-PCR) (Jeffreys et al. 1994) technique was developed to detect mutations at the DNA level directly in sperm cells thus avoiding the need to measure mutations arising in offspring. A minisatellite locus is amplified in multiple reactions, each containing a DNA equivalent to that from a number of sperm cells (usually <200), under conditions that are sensitive enough to detect the PCR product from a single DNA molecule. SP-PCR has been proven very efficient, remarkably free of PCR artefacts, and it can be used to measure mutation rates as low as 10^{-3}/cell. This method can overcome the small sample size limitation in pedigree analysis because there are millions of sperm in an average ejaculate and numerous PCR reactions can be run simultaneously. Mutation frequencies can be determined in individuals by SP-PCR, whereas pedigree analysis only gives an average from the population. A modification of SP-PCR, a single-molecule PCR, has been used to quantify the *in vivo* frequency of spontaneous and radiation-induced mutations in somatic and germ cells at mouse tandem repeat locus (*Ms6-hm*) (Yauk et al. 2002).

Unknown mutations can also be screened by the chemical cleavage of mismatches (CCM) (Moser and Dervan 1987; Cotton et al. 1988; Grompe et al. 1989). This method detects mismatches, variations in the DNA sequence, by a series of chemical reactions. Heteroduplexes formed between wild-type and variant DNAs are treated with the single-strand-specific S1 nuclease to cleave the DNA at the point of mismatched bases (Shenk et al. 1975). The differential

mobility of native and denaturated DNA-DNA heteroduplexes coupled with their differential melting temperature has been exploited by Myers et al. (1985a). CCM is a highly sensitive method that shows the position of the mutation by the size of the fragments generated. Quite large fragments (over 1 kb) can be analyzed. However, this method is experimentally difficult and since the necessary reagents are toxic, the usage of CCM is inconvenient. In the RNase mismatch cleavage method (Myers et al. 1985b; Winter et al. 1985) mismatches in RNA-DNA heteroduplexes are cleaved by RNase. This method does not reliably detect all mutations (Gibbs and Caskey 1987). However, nearly all single-base-pair mismatches can be detected by using sense and antisense probes (Myers et al. 1985c). Thus, this method may be used to identify the mutated base and its position in a heteroduplex. No toxic reagents are needed, but the disadvantage of this method is the poor quality of the results.

In addition to above mentioned mutation screening methods, single-strand conformational polymorphism (SSCP) analysis (Orita et al. 1989a, b) has also been widely used in mutation screening. It is based on a tendency of single-stranded DNA to fold up according to the DNA sequence in a non-denaturing gel. Any variation in DNA sequence (conformation) is seen in the gel as a mobility shift. SSCP analysis is suitable for relatively short PCR fragments, the optimal fragment size being approximately 150 bp (Hayashi 1992). To maximise the differential migration of DNA fragments in SSCP it is crucial to optimise the experimental conditions. Thus, the sensitivity of SSCP for mutation detection has been reported to vary from 30% to 100% (Michaud et al. 1992; Sarkar et al. 1992a; Claustres et al. 1993; Sheffield et al. 1993). SSCP analysis has proven to be a simple, cheap, and effective technique for detecting single base substitutions. However, it does not reveal the nature or position of any mutation detected. Dideoxy fingerprinting (ddF) (Sarkar et al. 1992b) is a hybrid technique that combines aspects of SSCP and dideoxy sequencing. Dideoxy fingerprinting analyses each band in a sequencing ladder by SSCP. It is based on changes in denatured nucleic acid size/mobility and it can detect the presence of single base and other sequence changes in PCR-amplified segments. Moreover, the point of the mutation can be estimated from the ddF pattern and samples with different mutations have different fingerprints. This method has been used to screen fragments of about 250 bp with virtually 100% sensitivity (Sarkar et al. 1992b), but it is complicated to interpret.

An even more efficient mutation detecting method than SSCP has turned out to be denaturing gradient gel electrophoresis (DGGE) (Fischer and Lerman 1980, 1983; Myers et al. 1987; Sheffield et al. 1989, 1992; Cariello and Skopek 1993). This method separates DNA molecules based on their sequence on a denaturant or a temperature gradient (Wartell et al. 1990) gel. Migration of DNA

continues until the DNA duplexes reach a gel position where the DNA strands melt and separate. After this, the denatured DNA does not migrate much further. The melting temperature of the fragment is determined by its DNA sequence. DGGE is often used in conjunction with PCR to determine if a given DNA fragment contains a mutation. In DGGE, the choice of the primers is critical for mutation detection. Primers with long stretches of Gs and Cs (GC-clamps) are used to maximise the sensitivity of the method for mutation detection (Myers et al. 1985d; Sheffield et al. 1989). DGGE has been shown to detect differences in the denaturing behaviour of small DNA fragments (200–700bp) that differ by as little as one base pair. Using optimized conditions, 100% of single-base substitutions, frameshifts, and deletions less than about 10 bp can be resolved from the wild-type sequence. However, GC-clamps in the primers make DGGE an expensive procedure and another disadvantage of the DGGE method is that it does not reveal the position of the mutation. An adaptation of DGGE, called constant denaturant gel electrophoresis (CDGE) (Hovig et al. 1991), is based on the electrophoretic runs of perpendicular gels (the electrical field is perpendicular to the linear gradient of the denaturing chemicals). In CDGE, a specific concentration of the denaturing chemicals is selected to give maximum separation between the wild type and mutant sequences of DNA fragments.

In addition to DGGE, heteroduplex analysis (Keen et al. 1991; White et al. 1992) can also observe single base pair mismatches. The heteroduplex gel mobility method is based on change in native nucleic acid conformation/mobility on nondenaturing gels following PCR. This method is very simple and cheap. However, its disadvantage is that it is applicable only to sequences <200 bp long and its sensitivity is limited. Heteroduplex analysis does not reveal the position of the change.

As in heteroduplex analysis, PCR products are also examined for mutations in denaturing high-performance liquid chromatography (D-HPLC) analysis (Oefner and Underhill 1995; Underhill et al. 1996). It is a form of reverse-phase ion-pair high-performance liquid chromatography. D-HPLC is used after PCR to divide heteroduplexes from homoduplexes in mixtures of wild type and mutated sequences. This method is based on change in native nucleic acid size/mobility. Instead of polyacrylamide gel matrix used in conventional heteroduplex analysis to separate homo and heteroduplex species in a non-denaturing environment, D-HPLC uses partially denaturing conditions to exaggerate the difference between the two species. D-HPLC is very efficient, accurate (100% of cystic fibrosis transmembrane conductance regulator gene mutations, Le Maréchal et al. 2001), and rapid as it can be automated. The disadvantages of this method are the need for expensive equipment and modification of PCR conditions because optimisation for each segment is required for full sensitivity of the method.

Among mutation screening methods, the protein truncation test (PTT) (van der Luijt et al. 1994) is a specific test for frameshifts, splice site, or nonsense mutations that truncate a protein product. This test is based on the size/mobility of the protein generated *in vitro* and it is highly specific, revealing the approximate location of any mutation. Large genomic regions can be tested using genomic DNA rather than RT-PCR. Several variants have been developed to give cleaner results, usually by incorporating an immunoprecipitation step. However, this method is expensive, experimentally difficult, usually needs RNA, and can detect only chain terminating mutations.

Mutation detection has become much more efficient with the development of a DNA microarray (DNA chip) (Cheung et al. 1999). Many different oligonucleotides are located in a DNA chip. The DNA microarray technologies employ a reverse nucleic acid hybridization approach. The probes consist of unlabeled DNA fixed to a solid support (the arrays of DNA or oligonucleotides) and the target is labelled and in solution. Many SNPs as well as mutations within these repeats can therefore be scored in a single experiment (Wang et al. 1998). Minisequencing can also be used in this mutation detection method. Oligonucleotide arrays are quick, have a high throughput, and they might detect and define all mutations. However, the equipment is expensive and only a limited number of genes can be analysed with these arrays.

As automated fluorescence sequencers have become standard items of laboratory equipments during the past few years, direct sequencing (Wong et al. 1987b) has become more common as a means of primary mutation screening. Sequencing detects all changes and the mutations will be fully characterized. However, sequencing is expensive and the sequences may be hard to interpret. Much time can be wasted investigating artefacts, especially if the sequencing template is not of the highest quality. Capillary electrophoresis (Simpson et al. 1998) is a powerful technique for DNA sequencing and fragment analysis especially when it is combined with confocal fluorescence detection, whereby it yields excellent sensitivity together with high speed and high-throughput, even fully automated separations. Using a four-colour detection system, DNA sequencing of 500 bp can be performed with 99% in less than 10 minutes. However, a limitation of this method is its resolution, thus making it only useful for the analysis of large fragments.

One of the most efficient mutation screening methods is mass spectrometry (MALDI-TOF MS) (Pieles et al. 1993). It is a strategy for analysing STR loci in the order of seconds per sample, with the advantage that it is not necessary to use allelic ladders. As part of the process, the products for analyses are co-crystallised with a small organic compound, the matrix. Irradiation with a short laser causes the spontaneous volatilisation of matrix molecules. Along with these

matrix molecules, DNA molecules are ionised and desorbed into the gas-phase, where they are subjected to an electric field. Determination of the molecular weight of the DNA is achieved by measuring the time-of-flight towards a detector, which is proportional to the mass. The main disadvantages of this method are the expensive equipment and the limitation in the size since only fragments of <100 bp can be analysed.

2.3.3 Equilibrium theory

Mutation and selection are the two basic forces in evolution. The equilibrium theory assumes that the population is in balance between spontaneous mutations and selection. Spontaneous mutations arise in every generation producing a new variation to the gene pool, and selection eliminates some of these mutations, depending on their effects on reproduction (= fitness). The effects of mutations on fitness vary. Some mutations cause death before reproduction and are therefore eliminated in one generation. Other mutations allow reproduction and can thus persist in the population for varying periods of time.

Radiation exposure can disturb the balance between mutation and selection by introducing new mutations into the gene pool of the population. Furthermore, these new, induced mutations are subject to selection. If the population is exposed to radiation in one generation only, there will be a transient increase in the mutation frequency in the first generation after irradiation followed by a progressive decline to the old equilibrium. If the mutation rate is increased as a result of radiation in every generation, the population will attain a new balance between mutation and selection at a higher mutation frequency. The time and the rate at which the new equilibrium is reached depend on the induced mutation rate and selection coefficients.

2.4 Mammalian gametogenesis

In the mammalian gametogenesis, primordial germ cells migrate into the embryonic gonad and engage in repeated rounds of mitosis (many more in males than in females, which may be a significant factor in explaining sex differences in mutation rates) to form oogonia in females and spermatogonia in males. Further growth and differentation produce primary oocytes in the ovary and primary spermatocytes in the testis. These specialized diploid cells can undergo meiosis. Meiosis involves two cell divisions but only one round of DNA replication, so the products are haploid.

In the mammalian male germline, stem cell spermatogonia are present throughout life. They recycle and produce later spermatogonial stages.

Spermatogenesis is a continuous process of mitotic and meiotic cell divisions, occurring from the beginning of sexual maturity. Male meiosis takes place only a few weeks before germ cell maturation producing four spermatozoa. In testes on adult males, all germ cell stages are present: stem cell spermatogonia and differentiating spermatogonia (pre-meiotic stages), spermatocytes (meiotic cells), and spermatids and spermatozoa (post-meiotic cells) (Searle 1974; Lyon 1981). In the male mouse, the number of cell divisions from zygote to sperm is of the order of 62 at age 9 months, assuming a 9-month generation (Drost and Lee 1995). The duration of spermatogenesis in the mouse is 42 days. From this, the time required for stem cell division is 8.5 days. The duration of human spermatogenesis is about 74 days (Figure 2). In the human male, there are 30 male cell divisions from zygote to spermatogonial stem cells at puberty (13 years). Six subsequent cell divisions are required for spermatogenesis, but thereafter the spermatogenesis cycle occurs approximately every 16 days or 23 cycles/year. This means that in human males, the number of cell divisions required to produce sperm is age-dependent. If an average age of 20 is taken as the male reproductive age, then the total number of cell divisions prior to sperm production is about $30 + [23 \cdot (20\text{-}13)] + 6 \approx 200$, increasing to ≈ 430 at age 30, ≈ 660 at age 40 and ≈ 890 at age 50 (Vogel and Motulsky 1997).

In the mammalian female germline, oocytes are already formed in late embryogenesis and remain arrested until the onset of sexual maturity. In females the cytoplasm divides unequally at each stage: the products of the first meiotic division are a large secondary oocyte and a small cell (polar body) and the secondary oocyte then gives rise to the large mature egg cell and a second polar body. The estimate for the number of germ cell divisions (from zygote to mature egg)/generation is 25 for mouse females (Drost and Lee 1995). In humans, primary oocytes enter meiosis I during fetal life but then arrest at the prophase stage right through to puberty or later (Vogel and Motulsky 1997). During this time, the primary oocytes complete their growth phase, acquiring an outer jelly coat, cortical granules, ribosomes, mRNA, yolk, etc. After puberty, one oocyte a month completes meiosis only a few hours before ovulation. Crossing-over in the female germline also occurs in the late embryonic stages. In human females, the number of cell divisions from zygote to fertilized oocyte is constant because all the oocytes have been formed by the fifth month of development (about 22 cell divisions) and only two further cell divisions are required to produce the zygote, irrespective of the age at which fertilisation occurs. The estimate for the number of germ cell divisions (from zygote to mature egg)/generation is thus 24 for human females (Vogel and Motulsky 1997).

It is established from studies with the mouse that there are major differences in the sensitivity of different germ cell stages in the male to ionising radiation

and a number of chemical mutagens when investigated with dominant lethal, translocation, specific-locus, or minisatellite mutation tests. Such sensitivity differences may reflect the characterictics of the different germ cell stages in terms of metabolism, chromosomal condensation, presence or absence of repair systems, etc. If the mechanisms of minisatellite mutation in females are similar to those in males (see 2.2.1.2), then minisatellite mutation in the maternal germline may only occur before birth. Thus, stem cell spermatogonia and immature, arrested oocytes are the stages most at risk of accumulating hereditary radiation damage (Searle 1987).

2.5 Molecular biological methods in genetic risk assessment

2.5.1 Methods currently available

The detection of changes in the germline mutation rate in human populations is extremely difficult. Traditionally used methods for evaluating the mutational load in human populations contain the search for untoward pregnancy outcomes, which includes stillbirths, neonatal deaths occurring during the first two weeks of life, and major congenital malformations. Malignancies, chromosomal aberrations, and electrophoretic protein variants have also been studied. Because of the very low frequency of spontaneous mutations at most loci, enormous sample sizes are required to detect de novo mutations or increases in mutation rate.

Spontaneous mutation rates in minisatellite are orders of magnitude higher than those of most protein-coding genes (see 2.2.1.3), and thus mutation rates can be studied by using reasonable sample sizes. The calculations use the number of rare events (mutations) in the control and exposed groups. Based on the Poisson distribution, the variance should be equal to the mean value. According to Poisson distribution the main statistics is:

$$R = (E - C) / \sqrt{(E + C)} , \qquad (1)$$

where: E = number of mutations in exposed group
C = number of mutations in control group

For a statistically significant difference between the two groups, this ratio should exceed a cut-point value derived from the Student distribution. For the reason of simplicity, R = 2 would roughly correspond to 95% probability. Thus, assuming the Poisson approximation, the sample size N needed for detection of

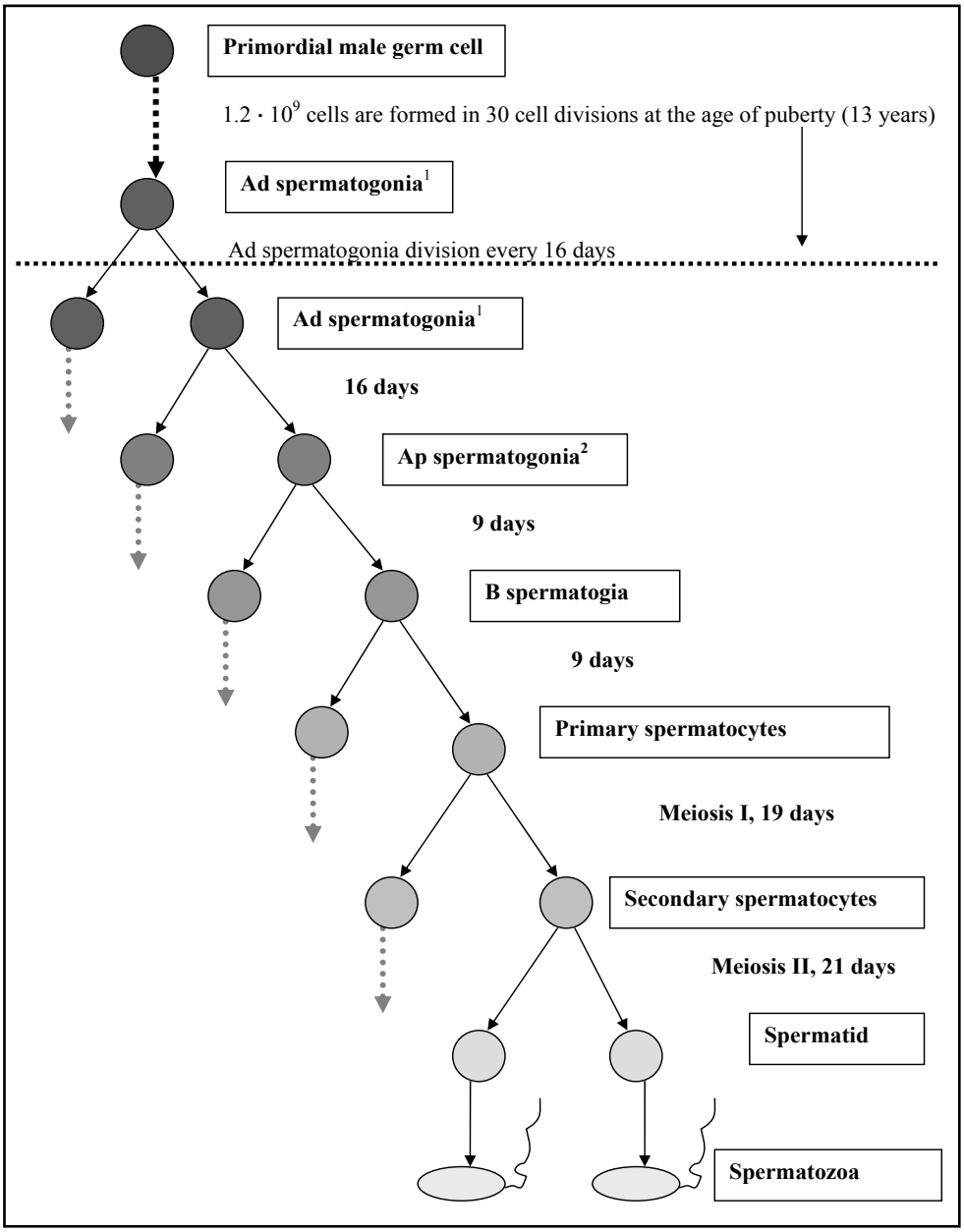

Figure 2. Human spermatogenesis (modified from Vogel and Motulsky 1997).
[1]Dark-staining A-type spermatogonia, [2]Pale-staining A-type spermatogonia.The number of cell divisions needed to produce a sperm cell = ~ 30 + [23 · (age in years-13)] + 6.

a statistically significant difference ($p < 0.05$) in the mutation rate between the two groups using the equation (1) can be calculated by the formula:

$$(E - C) / \sqrt{(E + C)} = 2 ,$$

where: $E = k \cdot N \cdot s \cdot m_0$

$C = N \cdot s \cdot m_0$

k = expected increase in mutation rate

N = sample size (number of offspring)

s = number of studied loci

m_0 = mean spontaneous mutation rate

$$, \text{ then } N = [4 (k+1)] / [(k-1)2 \cdot s \cdot m_0]. \quad (2)$$

When the expected increase in the mutation rate (k) is 2 (2-fold increase of the mutations), then according to Eq. (2):

$$N = 12 / s \cdot m_0 \quad (3)$$

Therefore, the sample size (N), which is necessary to detect a doubling (= 2-fold increase) of the mutation rate varies considerably (Table 3), depending upon the mutation detection method used, from 120 for minisatellite loci to 1 200 000 for protein-coding genes. According to these calculations minisatellites seem to be a superior method for detecting new mutations in the human genome.

Table 3. Minimal sample size (N) for detecting a 2-fold increase in the mutation rate [Eq.(3)].

Study system	Mean spontaneous mutation rate/locus (m_0)	Minimal sample size (N)[1]
Protein-coding genes	10^{-5} - 10^{-6}	120 000 - 1 200 000
Microsatellites	10^{-3} - 10^{-4}	1 200 - 12 000
Minisatellites	10^{-2}	120

In these calculations, s = 20 in each study system.
[1]Minimal sample size (N) contains an equal number of studied case and control offspring, e.g., 60 case offspring and 60 control offspring in the case of minisatellite loci.

2.5.2 Future perspectives

New molecular biology methods of revealing genetic damage in exposed human populations are needed in genetic risk assessment in the future. There is need for the more complete, efficient, and automated genetic screening of offspring. This could perhaps be achieved by using automated DNA sequencing or fluorescent in situ hybridization (FISH) methods. Since a single ejaculate of semen can contain 10^8 or more sperm, they are ideal monitors for the effects of radiation and other mutagens. They provide, in principle, a virtually limitless source of new mutant molecules derived from a given donor. Minisatellite mutations have already been explored from individual sperm cells using the SP-PCR method (see 2.3.2), but application of existing and the further development of individual sperm assays is needed. Unfortunately, human oocytes are not readily obtainable and thus the possibilities of obtaining genetic information directly from human oogonia or oocytes are minimal. Therefore, pedigrees remain the only source of maternal mutations.

Maternal mutations may be studied by minisatellite loci thus minimizing the number of offspring to be examined. However, the extensive amount of minisatellite (usually noncoding) DNA in eukaryotic genomes is a puzzle for molecular evolutionists. Since noncoding DNA has already been shown to affect transcription and protein binding at the RNA level, it may have a broad-ranging control function that has not yet been discovered. Noncoding DNA must have been maintained in evolution because in some (so far unknown) way, it is essential for cell survival. A challenge in the future of human genetics is to distinguish neutral variation in the human genome from functionally relevant DNA variation that influences individual differences in human biology.

In addition, further research and new methods are demanded in order to reveal the molecular mechanisms and pathways included in minisatellite mutagenesis as well as in other radiation-induced genetic damage. The actual nature of radiation-induced mutations has been poorly studied. It is known that a number of radiation-induced mutations are deletions, but very few loci have been investigated so far. Further work is required to examine a number of loci at which induced dominant mutations are frequent. They may help to show why some loci are highly mutable, while others seem to be completely immutable (Kohn and Melvold 1976; Arndt and Hwa 2004). Analyses of the effects of the genes known to play important roles in DNA turnover and their influence on recombination hot-spots rates and profiles using knockout mice should help defining the regulators of minisatellite instability. Yeast models have shown to be promising, but ultimately a manipulable mammalian model will be needed. Major challenges will be to understand the nature of the hypersensitivity of minisatellites to ionising radiation as well as the subsequent heritable instability.

2.6 Ionising radiation

2.6.1 Background

Humans are exposed throughout life to low-level ionising radiation from natural sources. This radiation comes from outer space (cosmic rays) and from radioactive substances in the earth's crust, (the most important ones are ^{238}U, ^{235}U, and ^{232}Th). In Finland, most natural exposure is due to indoor radon (^{222}Rn), a gas from the earth's crust. Radon gas, however, does not cause exposure of the gonads. The human body (muscles, bones, and other tissues) contain trace amounts of naturally occurring radioactive isotopes (the most important is ^{40}K). In addition to radiation from natural sources, most people receive additional exposure from manmade sources. These manmade sources consist mainly of medical radiation, but also commercial and industrial activities make their contribution to the total radiation exposure of humans. Radiation protection standards assume that radiation doses above natural background doses cause additional health risks, notably an increase in the induction of cancers. The increase in cancer incidence is assumed to be proportional to the additional doses.

2.6.2 Radiation quantities

The biological effects of ionising radiation are primary due to its ability to ionise the material in the cells that make up the body's tissues. Each discrete photon

or particle of ionising radiation has enough energy to knock electrons out of atoms and to break chemical bonds in the molecules from which human tissues are built. In general, radiation protection evaluates alpha (α)-, beta (β)-, gamma (γ)-, X-rays, and neutrons.

For radiation risk assessment, the health effects of α- and β-ray exposure appear only when α- or β-emitting materials are ingested or inhaled (i.e., internal exposure). Alpha (α) rays consist of posi-tively charged particles formed by two protons and two neutrons (= a nucleus of helium). Alpha rays are produced by spontaneous decay of certain radioactive isotopes (e.g., ^{226}Ra, ^{239}Pu, ^{240}Pu, ^{234}U, ^{235}U, ^{238}U, and ^{222}Rn). Because of its large mass and positive charge, a α-ray can usually pass only a short distance in water (µms) or air (<40 mm) and thus a single piece of paper or human skin can stop α-rays effectively. The beta (β) ray is a particle ray consisting of a fast electron (i.e., a negative charge) whose mass is about 1/2000 of the mass of a proton or neutron. Beta rays are produced by the spontaneous decay of certain radioactive isotopes (e.g., ^{3}H, ^{14}C, ^{32}P, and ^{90}Sr). Depending on its energy (= speed), a β-ray can travel between <0.1 mm and <10 mm (3H and ^{32}P) in water and up to 10 m in air. In general, a few mm thick sheet of aluminium will stop β-radiation.

Much more important than α- and β-rays in assessing the health effects of radiation are γ- and X-rays as well as neutrons. Gamma (γ) rays are electromagnetic waves similar to visible light, but the wavelength of γ-rays is much shorter (and their energy is far higher) than that of ultraviolet light. Gamma rays are produced following the spontaneous decay of certain radioactive isotopes (e.g., ^{60}Co and ^{137}Cs). As a ^{60}Co γ-ray can penetrate deeply into the human body, it has been widely used for cancer radiotherapy. Gamma rays can be stopped by thick walls of concrete or lead. X–rays have the same characteristics as γ-rays, although they are produced in a different way. When high-speed electrons hit metals, the electrons are stopped and energy is released in the form of an electromagnetic wave. X-rays consist of a mixture of different wavelengths, whereas γ-ray energy has a fixed value (or two) characteristic to the radioactive material. Neutron particles are released following nuclear fission (splitting of an atomic nucleus producing large amounts of energy) of uranium or plutonium. Neutrons are uncharged particles and they do not produce ionisation directly, therefore they do not damage cells. However, their interaction with atoms can give rise to α-, β-, or γ-rays which then produce ionisation. Since the human body contains large amounts of hydrogen (70% of human body constitutes of water molecules), ionisation can take place when neutrons hit the nucleus of hydrogen, i.e., a proton that is positively charged. Neutrons are penetrating and can only be stopped by thick masses of concrete, water, or paraffin. At equivalent absorbed doses, neutrons can cause more severe damage to the body than γ-rays. In male mice,

fission neutrons have been shown to be about 23 times more effective mutation inducers than γ-rays (Batchelor et al. 1966).

Several different dose estimates are needed in the assessment of radiation-induced risks in humans. The absorbed dose (D) of ionising radiation is the amount of energy absorbed/unit mass within the target (cell, tissue, organ, or human body). The unit of the absorbed dose is gray (Gy), which is defined as 1 J/kg. Even though 1 J/kg is a minor amount of energy, it is a high radiation dose because the biological effect of radiation is not based on the heating of tissue but on the break-up of important chemical structures. The basic assumption in radiation protection is that the harmful effects of ionising radiation are fundamentally related to the absorbed dose. The dose rate expresses the absorbed dose in a unit of time. The most used unit for a dose rate is Gy/h. The unit of the dose rate of the effective dose is Sv/h. The dose rate from natural background radiation is 0.04–0.3 µSv/h. Equivalent dose (H) describes the health effects of ionising radiation. It depends on the absorbed dose and the quality and energy of the radiation. The equivalent dose can be obtained by multiplying the absorbed dose with the weighting factor (w_R) for a particular radiation type. X-, γ-, and β-rays have a w_R of 1. Depending on their energy, neutrons have w_R of 2.5–10 (even 20). The highest w_R of 20 is used for α-particles. The unit of the equivalent dose is sievert (Sv). The concept of effective dose (E) (ICRP 60, 1991) is used to describe the total health effects of radiation doses on different organs or tissues. The effective dose can be obtained by multiplying the equivalent dose for each organ with the weighting factor (w_T) for that particular organ. The whole body has w_T of 1.00. The lowest w_T of 0.01 is used for skin and bone surfaces. Human gonads have the w_T of 0.20. The unit of the effective dose is also the sievert (Sv). The doubling dose (DD) is the radiation dose required to produce as many mutations as those occurring spontaneously in one generation (see 2.9.2). The genetically significant dose is the gonadal dose which, if received by every member of the population, would be expected to produce the same total genetic effect on the population as the individual doses actually received. An acute dose is a large radiation dose (>1 Gy) to the whole body delivered during a short period of time (few days at most). An acute dose can cause clearly identifiable symptoms in blood-forming organs and the gastrointestinal tract. A chronic dose is a relatively small amount of radiation received over a long period of time. In chronic exposure the human body has time to repair damage and therefore, chronic/low-dose-rate irradiation is only about one-third as effective in mutation induction as acute/high-dose-rate exposure.

Linear energy transfer and relative biological effectiveness are also important concepts in radiation risk assessment. Linear energy transfer (LET) indicates the energy deposited in matter along the track, i.e., the frequency of ionisations. LET

can differ considerably between different radiation types. In general, the larger the mass or charge of the particle or amplitude of electromagnetic radiation, the greater the LET. The relative biological effectiveness (RBE) can be obtained by dividing the dose from reference radiation (e.g., γ- or X-rays) by the dose from test radiation, which causes the same effect. Thus, RBE gives the ratio of the effectiveness/unit dose. RBE describes the ability of a given type of radiation to induce different biological endpoints, such as DNA damage, mutations, cell death, chromosomal damage, and cancer. The RBE is often different for acute, single exposure and for protracted or fractionated exposure. RBE can also vary depending on the endpoint chosen for examination.

2.6.3 Biological effects of ionising radiation on humans

The biological effects of ionising radiation are determined in terms of their effect on living cells. The target for radiation action is the entire human genome. Therefore, different genomic regions have equal initial probabilities of having radiation-induced biological effects, e.g., mutations. Radiation-induced biological effects are initialized by the random deposition of energy in the cells, causing the high-energy ionisation of atoms. Ionisation may affect DNA directly or indirectly (BEIR V, 1990). Direct effects arise from energy deposited directly on the DNA molecule. Ionising radiation induces base damage, single- and double-strand DNA breaks (SSBs and DSBs), and DNA-protein cross-links. Different types of mutations (see 2.6.4) might arise, as well as more severe forms of DNA damage, which prevent subsequent replication of the genome. In indirect effects, ionisation in other molecules (mostly water molecules) leads to the formation of reactive molecules (free radicals), which in turn attack the DNA.

Different defence mechanisms have been developed against these above-mentioned DNA attacks. The human body has defence mechanisms against damage induced by radiation as well as by chemicals. DNA damage is mostly efficiently repaired by cellular (DNA) repair processes. The final yield of mutations depends on the types of damage initially induced and on the efficiency of DNA repair processes. In a harmless situation, cells may be undamaged by the dose. However, even low radiation doses can damage or alter the DNA of irradiated cells. After the damage, either the damaged cells are repaired correctly and operate normally or they are repaired incorrectly and operate abnormally, thus resulting in biophysical changes (e.g., cancer). High radiation doses tend to kill cells by apoptotic or reproductive death and thus result in no residual damage. The biological effects of ionising radiation are of particular concern in the case of rapidly dividing embryo/foetus cells causing growth retardation, small head/brain size, mental retardation, childhood cancer, and even foetal death. Ionising

radiation can have different biological effects on humans depending on the type and length of radiation exposure. The biological changes may be manifested as medical symptoms that can be classified into deterministic or stochastic effects.

Of these above-mentioned radiation effects, the deterministic effects are based on extensive cell death. When only a few cells die, new cells replace them. However, when the radiation dose increases, a point will be reached where a sufficient number of cells are killed to affect the overall operation of the organ. The different types of radiation damage resulting from the loss of organ function are known as deterministic effects. Clinically observable deterministic effects, e.g., skin reddening and burning, radiation sickness, radiation pneumonitis, sterility (temporary or permanent), foetal damage and, cataracts, occur days to months, even several years (cataracts) after an acute radiation dose. Deterministic effects are most often seen in cases of high doses of radiation delivered over a short period of time (i.e., in the case of acute exposure in radiation accidents or radiation therapy) and they can always be connected with certain exposure. Deterministic effects occur only at doses above a certain threshold dose level. If the radiation dose is below this threshold dose level deterministic effects do not occur, but if the dose exceeds the threshold, these effects will certainly appear. The severity of deterministic effects increases sharply with increasing dose. Moreover, the period over which the dose was received has an effect on the severity of deterministic effect. If the dose is received over several weeks rather than all at once, the threshold dose at which the effect occurs increases considerably (usually by about 100%) and the deterministic effect will be smaller. The effect of a radiation dose on a particular person depends on biological factors (e.g., age and general health) as well as on chemical factors (e.g., the amount of oxygen present in tissue). Therefore, in any population there will be a range of sensitivity to radiation. Hence, the threshold dose in a given tissue will be reached at lower doses in more sensitive individuals. However, individual sensitivity has only limited impact on deterministic effects. As the dose increases more individuals will show the effect, up to a dose above which all exposed people will show the effect.

Not all radiation effects are deterministic effects as sometimes the radiation does not kill the cell but alters it in some way. In most cases, this alteration will not affect the cell significantly and there will be no observable effect. However, the cell may lose its ability to reproduce or there is a possibility that the injury might affect the control system of the cell. The effects of ionising radiation, which rely on chance, are called stochastic effects. These may be somatic (e.g., cancer) or hereditary effects (see 2.8.). They are based on DNA damage occurring after a long latency period (usually between 5-20 years) following radiation exposure. Hereditary effects and the development of cancer are the primary health concerns

attributed to radiation exposure. Any amount of radiation may pose a risk of causing cancer and hereditary effects. This risk is higher for higher radiation exposure. A linear-no-threshold (LNT) dose response relationship is used to describe the relationship between a radiation dose and the occurrence of cancer. This dose response model suggests that any increase in dose, no matter how small, results in an increase in risk. The likelihood of cancer occurring after radiation exposure is greater than a hereditary effect. For low levels of radiation exposure, the biological effects are so small that they may not be detected. For instance, it is not known how many cases of cancer are caused by natural background radiation. Stochastic effects are statistical effects based on genetic change in one exposed, surviving cell. Unlike deterministic effects, the amount of radiation exposure does not change the severity of the stochastic effect, but it does alter the chance of getting it. Thus, low radiation doses are also capable of inducing stochastic effects and the probability of stochastic effects is assumed to be linearly proportional to the dose without a minimum threshold dose. The total risk to an individual is determined by the cumulative radiation dose received during his/her whole lifetime and thus stochastic effects cannot usually be connected with a certain radiation exposure.

Currently, the human health risks associated with radiation exposure are based primarily on the assumption that the detrimental effects of radiation occur in irradiated cells and the biological effects of ionising radiation have been explained by the target theory. According to this theory, the critical target of radiation is the DNA molecule in the exposed cell. DNA damage occurs during or very shortly after irradiation of the nuclei in target cells. The radiation-induced changes are thought to be fixed already in the first cell division following the radiation exposure and the health effects are considered to result as a consequence of the clonal proliferation of cells carrying mutations in specific genes, causing hereditary effects (see 2.8.) or cancer.

These basic assumptions have recently been challenged by a growing research area based on the assumption that DNA is not the only target of radiation exposure. These radiation-induced non-targeted and delayed effects do not require direct radiation exposure and are particularly significant at low radiation doses. These effects include genomic instability and bystander effects.

In genomic instability (Kadhim et al. 1992; Morgan et al. 1996; Ullrich and Ponnaiya 1998; Wright 1998; Little 2000; Morgan 2003a, b), it has been observed that the progeny of irradiated cells show the occurrence of new mutations and/or new chromosomal aberrations or other genomic damage for many generations. The affected progeny also demonstrate a high level of lethal mutation, which may be measured as delayed reproductive cell death and/or delayed apoptosis.

These non-clonal, delayed effects occur in cells that were not exposed to radiation. Even very low doses of radiation are capable of inducing genomic instability and there is no linear dose response or dose rate effect. Genomic instability is induced both by high-LET and low-LET radiation, but not all cell lines show this effect. Animal studies indicate that some mouse strains are genetically more susceptible to the induction of genomic instability than others are. These mouse strains also show a higher susceptibility to radiation-induced malignancy. Genomic defects in the progeny are too common to result from a mutation in one gene and existing data suggests that stable non-mutational changes, epigenetic mechanisms and increased oxidative stress may play a role in radiation-induced genomic instability.

Moreover, in the bystander effect (Nagasawa and Little 1992; Prise et al. 1998; Belyakov et al. 2001; Bonner 2003; Morgan 2003a, b), mutations, changes in gene expression, and apoptosis are seen in cells that were not directly hit by radiation but were nearby. A bystander effect can be induced in unexposed neighbour cells by irradiated single cells through cell-to-cell communication (inhibition of cell communication prevents bystander effect) or via a culture medium in which irradiated cells have been grown earlier ('clastogenic factors'). The irradiation of cellular cytoplasm with either a single or an exact number of alpha particles has been shown to result in gene mutation in the nucleus of the cell. The induced mutations are point mutations instead of deletions, which is the form of damage usually seen after radiation exposure (see 2.6.4). It has been proposed that the bystander response is the initiating event in radiation-induced genomic instability (Morgan 2003c). It is likely that bystander effect is the main, but not necessarily the only, trigger of genomic instability. The bystander effect and genomic instability may be important modifiers of radiation response in the low dose region. Radiation-induced genomic instability and bystander effects may be inflammatory-type responses to radiation and they pose new challenges to evaluating the risk(s) associated with radiation exposure. The genomic instability and bystander endpoints are both transmissible (mutational) and non-transmissible (lethal). The balance of these in different cellular systems may lead either to an increased or decreased risk. Some scientists indeed argue that these non-targeted radiation effects are in fact part of the adaptive response to ionising radiation.

2.6.4 Radiation induced mutations

Ionising radiation can penetrate cells and create ions in the cell contents. These, in turn, can cause permanent alterations (mutations) in DNA. These are 'direct' mutations that have a linear dose response (Figure 3). Radiation-induced genomic

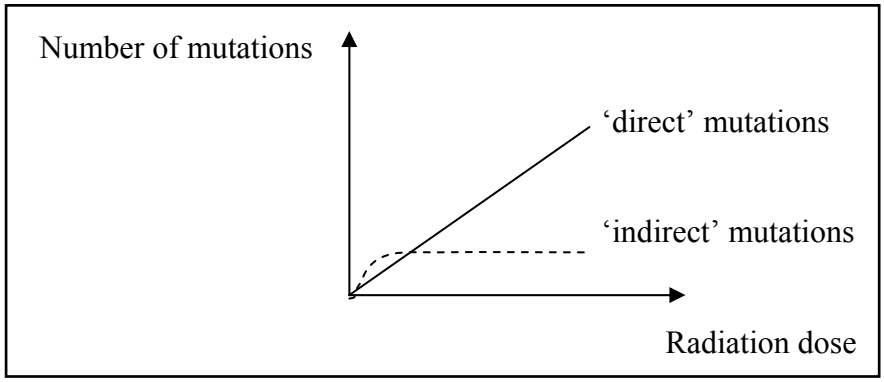

Figure 3. Radiation-induced mutations.

instability and bystander effects may cause 'indirect' mutations with no linear dose response (Figure 3). It has been speculated that these 'indirect' mutations are most important at very low doses of radiation exposure. In addition to radiation dose also the nature of the radiation exposure has an impact on the radiation mutagenesis. In the mouse, at the experimental doses used, it has been shown that chronic radiation is only about 1/3 as effective in producing mutations as acutely delivered radiation (Russell 1963).

It has long been known that ionising radiation induces both somatic and germinal mutations, and several mammalian cell mutational systems have been developed for studying chemical and radiation mutagenesis (Hsie et al. 1979; Moore et al. 1987). These include the endogenous hypoxanthine-guanine phosph oribosyltransferase (*hprt*), adenine phosphoribosyl transferase (*aprt*), thymidine kinase (*tk*), and the transgenic xanthine guanine phosphoribosyl transferase (*gpt*) genes. Various types of radiation-induced changes are broadly similar to those occurring naturally, e.g., point mutations, small and large deletions, complex rearrangements, etc. However, in well-studied experimental systems, most (e.g., 70-90% at *hprt* in CHO cells, Sankaranarayanan 1991a) radiation-induced mutations are recessive and due to DNA deletions. Radiation-induced mutations are often deletions that include not only a single gene, but also adjacent genomic regions, i.e., they are multilocus deletions (Sankaranarayanan 1991a, b; Thacker 1992). It can be presumed that they almost invariably cause loss-of-function which, in the case of dominant genes, occurs through haploinsufficiency (see 2.3.1.). The conclusions from molecular studies into the nature of radiation-induced mutations in mammalian (including human) somatic cells are also the same since most radiation-induced mutations are DNA deletions or due to other gross changes. However, the actual proportion of these mutations may vary with the test system and the locus under consideration.

Induction and recovery of these radiation-induced deletions can be interpreted on the basis of the current knowledge about DNA organization and functions in cells. Gene deletions require the occurrence of two DSBs in the DNA. There is evidence that the break points of radiation-induced deletions in the *hprt* gene of hamster cells are clustered (Morgan et al. 1990; Thacker et al. 1990). There are also hotspots for radiation-induced breaks in the chromosomes of blood lymphocytes of radiotherapy patients (Barrios et al. 1989; Holmquist 1992). These hotspots are in T-bands, which are very rich in both GC and Alu sequences (Holmquist 1992), suggesting that at least some radiation-induced deletions may arise by mechanisms similar to those inferred for naturally-occurring deletions mediated by Alu sequences. However, there are few mechanistic similarities between spontaneous and radiation-induced mutations, particularly for deletions. This is not unexpected, since ionising radiation produces mutations by the random deposition of energy in the cell, whereas spontaneous mutations arise through specific mechanisms.

Among radiation-induced mutations, point mutations constitute a small minority group with the exception of the bystander effect where the induced mutations constitute of point mutations. The low proportion of point mutations in the radiation-induced mutations is not easily explained. Using cultured mammalian cells it was shown that base substitutions are induced by ionising radiation, although multilocus deletions tend to predominate over point mutations in late cell generations. The detected base substitutions are usually those resulting in an inactive protein. Particularly in classic mouse studies, many substitutions remain undetected. These are likely to have effects that are more subtle than simple loss-of-function.

If human germ cell responses are similar as in the experimental studies, recessive deletion mutations will accumulate in the gene pool because they can be eliminated only when they are homozygous. As close inbreeding is exceptional in human populations nowadays, homozygosity for induced recessives is rare. Therefore, an assessment of the overall adverse health effects of induced recessives in the heterozygous condition is needed.

2.7 Naturally occurring hereditary diseases in man

2.7.1 Background

Naturally occurring hereditary diseases have been classically divided into Mendelian (or single gene) diseases, chromosomal diseases, and multifactorial diseases. The Human Genome Project has provided knowledge of the molecular

aspects of naturally occurring human hereditary diseases and the mechanisms of their origin. The identification of radiation-induced hereditary abnormalities in the human population is complicated by the quite high normal (background) incidence of such abnormalities. Various genetic and environmental factors underlie naturally occurring human diseases and the relative importance of these factors in the pathogenesis of different human diseases varies. Genetic factors dominate in Mendelian diseases and constitutional chromosomal anomalies; on the other end of the disease spectrum, environmental factors dominate in infectious diseases. Both genetic and environmental factors have a strong impact on the multifactorial diseases. Besides these classical disease types, new mechanisms of gene regulation and hereditary diseases have lately been detected. These new mechanisms include genomic imprinting, uniparental disomy, sytoplasmic inheritance, expansion of alleles, and mosaicism (UNSCEAR 2001). The effect of radiation on these new mechanisms is insufficiently known and thus they are not yet included in the assessment of radiation-induced hereditary risks.

2.7.2 Mendelian diseases

Mendelian diseases are also called single gene diseases because they are due to mutations in single genes showing predictable pattern of inheritance. There are five types of Mendelian pedigree patterns: autosomal dominant, autosomal recessive, X-linked dominant, X-linked recessive, and Y-linked. A disease is dominant if it manifests in the heterozygote and recessive if it manifests only in the homozygote. In addition to Mendelian diseases, genetic diseases in humans may be due to mitochondrial inheritance, which gives rise to a recognizable matrilinear pedigree pattern. Current estimates indicate that about 2.4% of children born alive are afflicted by one or another Mendelian disease (Sankaranarayanan 1998). Over 15 600 Mendelian disease genes or phenotypes have been established in the On-Line Mendelian Inheritance in Man (OMIM) Statistics (McKusick 2001) (Table 4). For many of these genes, extensive information is available, including sequence organization, the nature and function of the gene product, and the diseases associated with mutations in them.

Table 4. Number of entries in On-Line Mendelian Inheritance in Man (OMIM) statistics (McKusick 2001) (November 11, 2004) (http://www3.ncbi.nlm.nih.gov/Omim/mimstats.html).

	Auto-somal	X-linked	Y-linked	Mito-chondrial	Total
Gene with known sequence	9 397	416	48	37	**9 895**
Gene with known sequence and phenotype	358	38	0	0	**396**
Phenotype description, molecular basis known	1 483	136	1	26	**1 646**
Mendelian phenotype or locus, molecular basis unknown	1 297	132	4	0	**1 433**
Other, mainly phenotypes with suspected Mendelian basis	2 173	154	2	0	**2 329**
Total	**14 704**	**876**	**55**	**63**	**15 704**

When Mendelian genes and their function became better known, the classical 'One mutation, one disease' concept turned out to be too simplistic. Not all individuals carrying the mutant gene always express the mutant phenotype (variable penetrance) or are affected to the same degree (variable expressivity). Even in simple Mendelian diseases, mutations in the same gene may cause different phenotypes (allelic heterogeneity) (e.g., Charcot-Marie-Tooth disease and cystic fibrosis). Mutations in different genes can result in the same or nearly the same phenotypes (non-allelic heterogeneity or genetic heterogeneity) (e.g., Osteogenesis imperfecta and Ehlers-Danlos syndrome type VII). Identical mutations have also been reported to cause large differences in clinical severity (e.g., sickle-cell anaemia and cystic fibrosis).

2.7.3 Chromosomal diseases

Chromosomal diseases are caused by structural or numerical chromosomal abnormalities [e.g., partial deletion of 5p: *cri-du-chat* -syndrome (OMIM 123 450); trisomy for chromosome 21: Down syndrome (OMIM 190 685)]. Chromosome mutations, such as deletions and translocations, will usually lead to early fetal loss if they are unbalanced. Various chromosomal diseases are known in the human population and about 0.4% of children born alive are afflicted with detectable chromosomal diseases (UNSCEAR 2001). The microdeletion

syndromes (contiguous deletion syndromes) are conditions resulting from deletions of multiple, often functionally unrelated, yet physically contiguous loci. True microdeletion syndromes are caused by the haploinsufficiency of several loci. These loci are compatible with viability in heterozygous condition. They are identified clinically through a characteristic association of unusual appearance and defective organ development (e.g., Langer-Giedion syndrome, OMIM 150230). Mental and growth retardation are often prominent features.

2.7.4 Multifactorial diseases

Multifactorial diseases are common conditions (e.g., cleft lip, cleft palate, and neural tube defects) caused by complex interactions between genetic and environmental factors. They have a genetic component and they tend to cluster in families, but the inheritance pattern is not a simple Mendelian one. Most of the common diseases with onset predominantly in adults (e.g., diabetes mellitus, coronary hearth disease, allergy, asthma, most cancers, etc.) are also multifactorial diseases. The multifactorial diseases constitute the predominant load of genetic diseases, as at least 71% of children born alive will have multifactorial diseases [i.e., 6% congenital abnormalities (Czeizel and Sankaranarayanan 1984) and 65% common chronic diseases (Czeizel et al. 1988)]. Because of their high prevalence, they contribute very substantially to morbidity and mortality in human populations.

In multifactorial diseases the risk to first-degree relatives is much less than what is known for Mendelian diseases. In the multifactorial threshold model, it is assumed that all genetic and environmental causes can be combined into a single continuous variable called liability. Numerous genetic and environmental factors act additively, each contributing a small amount of liability and thus, its distribution is normal (Gaussian). Affected individuals are those whose liability exceeds a certain threshold. This threshold is exceeded by a larger proportion of population consisting of relatives of affected individuals than of the general population, i.e., the disease prevalence is higher among relatives of affected individuals than in the general population

2.7.5 Spontaneous mutation rates in humans

Mutagenic products of metabolism and the intrinsic instability of DNA (e.g., deamination, depurination, depyrimination, endogenous methylation, oxygen metabolism) can cause spontaneous damage to DNA. This leads in some cases to various types of spontaneous mutation (see 2.3.1) through errors in DNA repair, replication, and recombination processes (Ames 1989, Smith 1992). In general, the

mutation rate of a given gene is a function of the size and organization of the gene, the mechanisms involved, and the number of mutational sites. Most spontaneous mutations arise as a consequence of DNA replication errors. Therefore, the male germline has a higher mutation rate than the female germline due to continual post-pubertal mitotic processes in the male (Hurst and Ellegren 1998) (see 2.4). The likelihood of germinal mutations increases in older males (paternal age effect) (Crow 1993, 1997, 1999; Vogel and Motulsky 1997).

New mutations arise spontaneously in every generation. The frequency of diseases in the population is nevertheless believed to remain stable from one generation to the next (the equilibrium theory, see 2.3.3). In general, selection against autosomal dominant diseases is strong and thus they occur at similar frequencies in most populations. Most of them are due to new mutations. In autosomal recessive diseases selection pressures are much less efficient. Most of the mutant genes are carried by asymptomatic heterotzygotes, and these diseases can appear at high frequencies in some isolated populations.

Based on population studies, Vogel and Motulsky (1997) estimated that mutation rates for human genes vary from 10^{-4} to 10^{-6}/gamete/generation. However, the spontaneous mutation rates of tandem repeat sequences are much higher than in human regular genes. Minisatellites have a spontaneous mutation rate of 10^{-1}–10^{-2}/locus/gamete/generation (Buard et al. 1998), and microsatellites have a spontaneous mutation rate of 10^{-2}–10^{-4}/locus/gamete/generation (Ellegren 2000).

2.8 Hereditary effects of radiation

2.8.1 Background

Hereditary effects of radiation are those effects observed in offspring born after one or both parents have been exposed to radiation before the child was conceived. Hereditary effects are the result of a mutation and chromosome structural changes produced in the reproductive cells of an exposed individual. They should not be confused with the possible harmful effects due to exposure of the mother during a pregnancy. Radiation exposure of the parents can lead to lethality, malformations, and genetic diseases in future generations. Hereditary effects may appear in the exposed person's direct offspring, or several generations later, depending on whether the altered genes are dominant or recessive. Hereditary effects must be compatible with germ cell survival and division for transmission to the next generation, unless they are induced in non-dividing forms such as spermatozoa. As these effects can be caused only when ionising radiation reaches

the germ cells; penetrating X- or γ-rays (see 2.6.2) can induce germline mutations from outside the body, but α- or β-particles of short track-length must be present in the gonads and very close to the nuclei of germ cells in order to have a hereditary effect. The cells of special concern in heredity risk assessment for radiation are those most at risk of accumulating genetic damage, namely spermatogonial stem cells and resting oocytes (Searle 1987).

It has been shown that radiation increases the mutation rate in different organisms, including humans. However, many mechanisms (e.g., DNA repair, mutation fixation, transmission of mutant-bearing germ cells, as well as zygotic, embryonic, and postnatal survival of mutants) influence the frequency of mutations observed. Hereditary mutations range from harmful to beneficial. Humans evolved to the present state by utilizing beneficial mutations. Mutations with damaging effects are gradually eliminated from a population by natural means, since affected individuals are less likely to have offspring than normal individuals are. The more severe the damage produced by a given mutation, the more rapidly it will be eliminated. Mildly damaging mutations may require several generations before they gradually disappear. As mutations in persons without offspring have no genetic effects, the genetically significant dose of radiation to a population is markedly less than the total dose. The risk of hereditary effects caused by radiation is smaller (1.3%/Sv) than the risk of a lethal, radiation-induced cancer (10%/Sv in high, acute dose or 5%/Sv in low, chronic dose) (ICRP 1990). Therefore, the risk of a hereditary defect in humans has been estimated as 15/million births for a radiation dose of 1 mSv (UNSCEAR 2001). This is small figure compared with the risk of a spontaneous hereditary defect in humans from all causes that is of the order of several percent (see 2.7). As experimental data is not feasible for humans, results from mammalian mutagenesis experiments provide the most precise estimate of the induced mutation rate in germ cells of humans (UNSCEAR 2001). Thus, mammalian mutagenesis results have been used in an extrapolation of the genetic effects of radiation exposure in humans.

2.8.2 Mouse studies

The genetic effects of radiation have been extensively studied in a wide spectrum of biological systems (Sankaranarayanan 1982; DeMarini et al. 1989). The main purpose of mammalian mutagenesis experiments has been to elucidate those factors important in mutation induction in the germ cells of mammals as a model for humans. It is clear that the successful mutation test systems (e.g., 7-locus system and minisatellites) have been those in which the studied genes and their genomic loci were not essential for survival. In these cases, induced mutations

can be recovered and studied. Even viability-compatible changes may occur in regions that cannot be detected, so their phenotypes remain unknown. The least successful test systems, the histocompatibility (*H*)-loci in the mouse (Bailey and Kohn 1965; Kohn and Melvod 1976; Kohn et al. 1976) and ouabain resistance in mammalian somatic cells (Arlett et al. 1975; Thacker et al. 1978), are those in which the genes themselves are essential for survival and/or are located in gene-rich regions of the genome and for that reason cannot tolerate induced deletions. All the methods are effective in screening for mutations, although the differences in the number of offspring needed for screening reflect the relative ease with which offspring may be examined for different genetic endpoints. The 7-locus test and minisatellites require the least investment of time and resources, followed by the dominant cataract test, the enzyme-activity assay, and finally the protein-charge assay.

2.8.2.1 Traditional studies

The traditional mouse study method, the specific-locus (7-locus) method was developed by Russell (1951). It is the most efficient method of screening for transmitted germ cell mutations. It has provided virtually all experimental data on factors affecting the mutation rate in the germ cells of mam-mals. Briefly, the method involves mating homozygous wild type treated or control mice with untreated tester stock mice, homozygous recessive at 7 marker loci: *a* (non-agouti), *b* (brown), *cch* (chincilla), *p* (pink-eyed), *d* (dilute), *s* (piebald-spotting), and *se* (short ear), located in chromosomes 2, 4, 7, 7, 9, 14, and 9. Six of these loci control coat colour and the *se* locus affect the size of the external ear. The 7-locus method and its results in the mouse have been discussed in detail (Searle 1974; Selby 1981; Ehling and Favor 1984). Over a million animals were needed (over decades) to obtain meaningful results in the 7-locus method, nevertheless the results were difficult to interpret. It was shown that different types of mutations differed widely in their sensitivity to radiation. Even among the small number (n = 7) of genes studied, there is more than a 30-fold difference in their observed radiation mutabilities, with the s locus being the most mutable and the a locus the least mutable (Russell and Russel 1959; Russell 1965). Doubling of the natural background mutation rate required radiation doses of 0.1–2 Gy. Homozygosity tests uncovered the existence of recessive mutations or deletions in developmental genes, i.e., heterozygotes do not manifest the abnormal developmental phenotype (Rinchik and Russell 1990). Radiation dose, dose rate, dose fractionation, dose-fractionation interval, radiation quality, and germ cell stage affected the radiation-induced mutation rate. The consequences were minimised by allowing some time to elapse between irradiation and conception. Doses spread over a period of time (fractionated dose) produced a smaller effect

than if they were delivered all at once. The effects for a given dose were greater for the offspring of the irradiated male.

In addition to 7-locus test, a number of procedures have been developed to screen for induced dominant mutations in germ cells of the mouse. These include congenital malformations (Searle 1974; Kirk and Lyon 1982, 1984; Lyon and Renshaw 1988; Favor 1989), skeletal abnormalities (Ehling 1965, 1966, 1985; Selby and Selby 1977, 1978), cataracts (Kratochvilova and Ehling 1979), and growth retardation (Cattanach et al. 1993, 1996) as well as mutations that affect quantitative traits and fitness (Green 1968). Procedures have also been developed to screen for protein-charge (Soares 1979; Johnson and Lewis 1981; Pretsch et al. 1982; Peters et al. 1986) or enzyme-activity mutations (Bishop and Feuers 1982; Feuers et al. 1982) and thus provide methods to recover mutations with direct homology to human genes that can be characterised through biochemical methods. Mouse data support the view that radiation-induced, predominantly multi-locus deletions in different chromosomal regions are associated with growth retardation and dysmorphic features. Most of the mutational events scored are of the recessive loss-of-function type, involving the marker and several neighbouring genes. Thus, induced mutations could be identified through the phenotype of the marker genes and recovered for further studies.

The mutability as determined by the various genetic endpoints differed. The mutation rates for recessive 7-locus alleles and enzyme-activity alleles were similar and greater than the mutation rates to dominant cataract and protein-charge alleles. The type of mutation event scored by a particular test can determine the mutability of the genetic endpoint screened. When the loss of a functional gene product can be scored in a particular mutation test, as in the recessive 7-locus and enzyme-activity tests, a wide spectrum of DNA alterations may result in a loss and a higher mutation rate is observed.

2.8.2.2 Minisatellite studies

Minisatellites (see 2.2.1) have been identified and characterized in various genomes. In particular, systematic isolation has identified minisatellites in the mouse, rat, and pig genomes. Unstable mouse minisatellites originally identified by DNA fingerprinting are now known to consist of long arrays (0.5–16 kb in length) of short (4-6 bp) repeat units that are almost exclusively sequence-homogeneous with the longer arrays exhibiting the highest mutation rates (Kelly et al. 1991; Gibbs et al. 1993b; Bois et al. 1998, 2001). These properties distinguish these loci from true mouse minisatellites (0.5–10 kb, repeat size of 14–47 bp) (Kelly et al. 1989), and therefore these shorter mouse repeat sequences have been renamed expanded simple tandem-repeat arrays (ESTRs) (Bois et al. 1998). Differing from human minisatellites, mouse ESTRs are not preferentially associated with

centromeres or telomeres. Rather they are mainly found at interstitial sites (Jeffreys et al. 1987). ESTRs are the most hypermutable sequences isolated in the mouse genome, with germline mutation rates as high as 13%/locus (Yauk et al. 2002). However, in sharp contrast to human minisatellite loci, most mouse minisatellite loci mutate at a rate below 10^{-3}/gamete/generation and mainly by simple and intra-allelic mutation events, similar to those observed in human peripheral blood DNA (Bois et al. 2002). Unlike unstable human minisatellites, which mutate almost exclusively in the germline, mouse ESTRs show repeat instability in both germinal and somatic DNA. Therefore, there seems to be a fundamental difference between humans and mice in the turnover mechanisms at minisatellite loci and ionising radiation appears to induce instability at various types of hypermutable tandem-repeat arrays.

The hereditary effects of radiation have been studied experimentally using the mouse ESTRs. The probable selective neutrality of ESTR mutants should ensure that mutants are not lost prenatally. Several studies have shown that ionising radiation causes a significant increase in the germline mutation rate at the mouse ESTR loci (Dubrova et al. 1993, 1998a, b, 2000; Sadamoto et al. 1994; Fan et al. 1995; Niwa et al. 1996; Niwa and Kominami 2001; Barber et al. 2002; Dubrova and Plumb 2002). Attempts to transfer minisatellite instability to mice using large transgenic constructs ($\sim$30 kb) containing human hypermutable loci have failed to recapitulate the complex recombination-based germline instability observed in humans (Bois et al. 1997; Buard et al. 2000). It is possible that the transgenic constructs used failed to provide the correct chromatin environment needed for human minisatellites associated with the flanking recombination hotspots. However, more promising result has been obtained with microsatellite expansion of CGG repeat causing human fragile X syndrome. Baskaran et al. (2002) have generated transgenic mice lines that show a dramatic increase from 26 to >300 repeats in three generations. Their results support a postzygotic mechanism for CGG expansion that is independent of a genomic imprinting effect.

In mouse minisatellite studies, results concerning the relative radiosensitivity of different stages of spermatogenesis have varied depending on the study design. Dubrova et al. (1993) conducted a pilot study to determine the inducibility of ESTR mutations in mouse germ cells by radiation. Male (101/HY x C3H/SnY) F_1-hybrid mice were given an acute exposure of 0.5 or 1 Gy of γ-radiation using ^{60}Co and mated to untreated F_1 females six weeks later. Paternal ESTR mutations at loci 33.6 and 33.15 appeared to be induced in spermatogonia and stem cells by low doses of ionising radiation, with an estimated doubling dose of 0.5 Gy. However, the frequency of observed mutations did not appear to increase linearly with radiation dose because the mutation frequencies in

exposed mice in relation to controls were 1.9 and 1.6 for mice irradiated with 0.5 and 1 Gy.

Conflicting results were obtained by Sadamoto et al. (1994) who investigated ^{60}Co gamma radiation-induced germline mutations at ESTR loci. In this study, male mice (C3H/HeN) were exposed to 3 Gy of γ-rays and mated to female mice of strain C57BL/6N. The males were mated again with unirradiated female mice at different time intervals after irradiation to score minisatellite mutations at locus *Ms6-hm (Pc-1)* in different stages of spermatogenesis. Post-meiotic spermatids seemed to be much more sensitive to radiation-induced ESTR mutations than, e.g., spermatogonia and spermatozoa (paternal mutation frequencies were 28%, 15%, and 13%, respectively). The induced mutation frequencies were too high to be accounted for by the direct action of radiation on the locus and therefore, no reliable relationship between radiation dose and mutation frequency was seen. The authors described a small but statistically significant increase in the maternally derived *Ms6-hm* allele in addition to the paternally derived allele.

The experimental design of Sadamoto et al. (1994) was essentially repeated by Fan et al. (1995). They exposed male C3H/HeN mice to various doses (1, 2, or 3 Gy) of ^{60}Co γ-rays and mated them to unirradiated C57BL/6N female mice. The matings were done immediately, 3 weeks, or 11 weeks after irradiation for analysis of radiation effects on the spermatozoa, spermatid or spermatogonia stage germ cells. The spermatid stage was detected most sensitive to radiation and a statistically significant dose response was observed. The mutation frequency of the paternal allele increased linearly up to 2 Gy where the curve reached a plateau. The doubling doses of the paternal allele of *Ms6-hm* were 6, 0.79, and 6 Gy for the spermatozoa, spermatid and spermatogonia stages, respectively. Also these authors detected a small but statistically significant increase in the maternally derived *Ms6-hm (Pc-1)* allele in addition to paternally derived allele.

As in the two previous studies, spermatid stage germ cells were observed to be the most sensitive to radiation also by Niwa et al. (1996). Male C3H/HeN mice were exposed to ^{252}Cf radiation (35% γ-rays and 65% neutrons) and mated to unirradiated C57BL/6N females immediately, 2 weeks, or 10 weeks after irradiation corresponding irradiation of spermatozoa, spermatid, or spermatogonia stage germ cells, respectively. Paternal mutations at minisatellite locus *Ms6-hm* increased to 18%, 26%, and 24% when spermatids were irradiated by 0.35, 0.7, and 1.02 Gy of ^{252}Cf radiation, respectively.

Dubrova et al. (1998a) studied germline mutation induction at minisatellite loci *MMS10, 33.15, Ms6-hm*, and *Hm-2*. Male CBA/H mice were given whole-body acute X-ray irradiation of 0.5 and 1 Gy. Males were mated to untreated CBA/H females 3, 6, and 10 weeks after irradiation, corresponding induction of mutations

at these ESTR loci in pre- and post-meiotic stages of mouse spermatogenesis. The results showed a linear dose-effect relationship for mutations in pre-meiotic spermatogonia and stem cells, with a doubling dose of 0.33 Gy. There was no evidence for induced mutations in post-meiotic spermatids. The results could also be interpreted in such a way that the mutations result from damage accumulated in germ cells prior to meiosis. This does not necessarily indicate that the mutational events themselves occur pre-meiotically rather than later, e.g., in meiosis. Since meiotic stages were not included in this study, no firm conclusions could be drawn. Paternal irradiation did not affect the maternal mutation rate. These results were later repeated by using the mouse minisatellite loci *Ms6-hm* and *Hm-2* (Dubrova et al. 1998b).

In their following mouse study Dubrova et al. (2000) gave a total whole-body low-LET chronic γ-irradiation of 0.5 and 1 Gy using a ^{60}Co source. Also, whole-body high-LET chronic irradiation (0.125, 0.25, or 0.5 Gy) was performed using a ^{252}Cf source (67% neutrons and 33% γ-radiation). After irradiation male CBA/H mice were mated to untreated females of the same mouse strain 10 weeks post-irradiation. The F_1 mice generated were therefore conceived with sperm derived from irradiated stem cells. Radiation-inducted minisatellite mutations were scored at minisatellite loci *MMS10, Ms6-hm,* and *Hm-2*. Both types of radiation produced linear dose response curves for mutation of the paternal allele. The authors estimated the relative biological effectiveness (RBE) of neutrons as 3.36 (95% CI 1.30-5.41). They concluded that minisatellite mutation induction patterns were similar for both irradiations, indicating that the elevated mutation rate was independent of the ability of the cell to repair damage induced immediately or over a period of up to 100 hours. The paternal exposure had no effect on mutation rates of the maternal allele.

However, results by Niwa and Kominami (2001) revealed a hereditary risk to the maternally derived genome due to the paternal radiation exposure. They demonstrated a statistically significant increase (from 9.8% to 20%) in the maternal allelic mutation rate in F_1 mice born to at the spermatozoa stage irradiated male parents was demonstrated by Niwa and Kominami (2001). Partial body irradiation to the testicular portion was performed on male C3H/HeN mice. A total of 6 Gy of γ-rays was delivered using a ^{60}Co source. The irradiated males were mated to unirradiated females (C57BL/6N) immediately or 15-20 weeks after exposure to assess the effect of spermatozoa or spermatogonial stem cell irradiation. Paternal minisatellite mutations at the *Ms6-hm (Pc-1)* locus increased from 8.4% to 22% and 19% when spermatozoa and spermatogonia stages were irradiated. The authors concluded that as a consequence of male (sperm) irradiation, genomic instability is triggered in zygotes and in embryos of subsequent developmental stages, which then induces untargeted mutations

at the paternally derived allele *in cis* and the maternally derived unirradiated allele *in trans*.

More recently, both pre-and post-meiotic exposures were reported to increase mutation yield in two generations (Barber et al. 2002), suggesting that strains of mice may differ in stage susceptibility. Male CBA/H and C57BL/6 mice were given a 0.4 Gy whole-body chronic irradiation of fission neutrons by using a ^{252}Cf source. In addition, whole-body acute 2 and 1 Gy irradiation of X-rays was given to other CBA/H and BALB/c males. All males were mated to unirradiated females of the same inbred mouse strain; CBA/H males 3 and 6 weeks after irradiation and C57Bl and BALB/c males 6 weeks postirradiation. Minisatellite mutation rates were examined among F_2 and F_3 offspring at loci *Ms6-hm* and *Hm-2*. The authors demonstrated that rates of spontaneous mutations as well as radiation-induced trans-generational instability varied between three mouse strains examined (BALB/c >CBA/H >C57BL/6). Higher mutation rates did not correlate with allele length and they were possibly due to functional differences at cyclin-dependent kinase inhibitor 2a (*Cdkn2a*, OMIM 600 160) and DNA-dependent protein kinase catalytic subunit (*Prkdc*, OMIM 600 899) genes in BALB/c. Barber et al. (2002) concluded that the persistence of elevated mutation rates in the germline of two consecutive generations excluded the possibility that transgenerational effects were caused by radiation-induced mutations at any specific set of genes (e.g., DNA-repair genes) in the exposed F_0 male mice. The authors stated that significantly increased mutation rates in the germline of both generations clearly implicated an epigenetic mechanism for the transgenerational minisatellite instability.

The frequency of induced mutation appear to be significantly greater than the predicted frequency of radiation-induced DNA damage at a given minisatellite locus. The above-mentioned doubling (= 2-fold increase) of the minisatellite mutation rates at 0.5 Gy dose (Dubrova et al. 1993) is in discordance with the fact that at a 0.5 Gy dose only 50 double strand breaks (DSBs) have been shown to be generated/cell (Frankenberg-Schwager 1990). It is improbable that most of these breaks would have occurred in the few tandem repeats analysed since damage caused by ionising radiation appears random. Later estimation of doubling dose of 0.33 Gy by the same authors (Dubrova et al. 1998a, b) creates an even more unrealistic situation. This has led many investigators to conclude that the increased mutation rates observed at these ESTR loci are not necessarily the result of directly induced DNA damage at the specific loci but rather result from radiation-induced damage elsewhere in the genome or cell (Sadamoto et al. 1994; Fan et al. 1995; Dubrova et al. 1993, 1998a, b, 2000; Niwa and Kominami 2001; Barber et al. 2002; Yauk et al. 2002). The mechanism by which the repair of DSBs generates specific increases in tandem repeat instability can only be speculated.

Bois (2003) has described three possible effects, two *in cis*, and one *in trans*. One possibility is that DSBs generated in meiotic cells are by strand conversion quickly turned into Holliday junctions, which could then generate DSBs in the vicinity of, or within, tandem arrays. A second possibility could be that tandem repeats are particularly sensitive to long-range chromatin modifications such as change in DNA tension or relaxation subsequent to ionising radiation exposure. A third possibility is an effect *in trans* in which DSBs activate genomic defence mechanisms that could indirectly increase minisatellite instability. If ESTR mutations are non-targeted events, then there appears to be an indirect induction mechanism whereby radiation is sensed and in some way transduced into a signal that somehow destabilises tandem repeat loci. If correct, this would represent a completely novel mechanism for radiation mutagenesis.

2.8.3 Human studies

In a variety of experimental systems, the phenotypes of radiation-induced mutations in the specific genes were similar to those of spontaneous mutations. Even though animal studies have clearly shown a relationship between radiation exposure and hereditary effects, the real quantity of the hereditary effects in humans is so far unknown. Assessment of radiation-induced hereditary risks is a formidable task in the human population. Each of the human 30 000 genes can mutate in the germ
line and give rise to hereditary effects. Therefore, the spectrum of the possible effects is wide. The type and severity of radiation-induced hereditary effects can be highly different and there are still many open questions concerning hereditary effects of ionising radiation. Neither the extent to which a certain radiation dose increases the mutation frequency in the human population nor the health effects of this increase on the future generations are known. No direct evidence of radiation-induced hereditary effects has been detected in human populations – not even among the offspring of survivors of the atomic bombing of Hiroshima and Nagasaki (Neel et al. 1990, 1998; Otake et al. 1990; Byrne et al. 1998). Thus, radiation risk assessment is based on the general knowledge of human he-redity and on animal studies. It seems that humans are not as sensitive to hereditary effects as laboratory animals are (Neel et al. 1990). This may be due to strong negative selection in the critical periods during embryo and foetal development.

In contrast to animal studies, most of the human genes in which induced mutations have been examined, do not seem to be non-essential for survival. Thus, the failure to find induced germline mutations in humans is not due to the resistance of human genes to induced mutations but due to the structural

and functional constraints associated with their recoverability in live births (UNSCEAR 2001). It is difficult to measure changes in the human mutation rate because the majority of mutations will not express themselves in an individual as they are in a heterozygous state. Moreover, most of the mutations that occur are minor (e.g., a less efficient digestive system or a predisposition to a given disease) and therefore difficult to measure. The situation becomes even more complicated by the fact that they are often also impossible to distinguish from conditions caused by other influences. Recent studies of minisatellite loci (see below) are conducted in non-essential genes, but they are difficult to interpret because these mutations are perhaps induced by some indirect effects of radiation on these genes.

2.8.3.1 Hiroshima and Nagasaki

The most comprehensive study of heritable genetic effects following preconception irradiation in humans is that of the Japanese atomic bomb survivors. The main source of data is the Life Span Study (begun in 1950) of the survivors (n ~ 120 000) of the atomic bombings at Hiroshima and Nagasaki in Japan 1945. The atomic bomb survivors had a range of radiation doses of acute gamma and neutron irradiation. Their germinal doses varied between 360 and 600 mSv (average ~ 400 mSv), and 1 500 parents had doses greater than 1 Sv (Yoshimoto et al. 1990). However, nowadays the mean dose estimation of <200 mSv is used for this cohort (Pierce et al. 1996). The atomic bomb survivors showed a pattern of increasing mortality with an increasing dose for leukaemia and most solid cancers, with a significant increase following acute doses in the range of 200–500 mSv. Significantly increased cancer risk and a significant dose response has been seen at low doses (<100 mSv) (Pierce et al. 1996). In addition, the incidence of myocardial infarctions has been shown to increase among younger, heavily exposed persons (Wong et al. 1993; Shimizu et al. 1999).

The Hiroshima and Nagasaki cohort is ideal for studying hereditary risks at low radiation doses. To date those exposed to radiation during the bombings have had more than 80 000 children and tens of thousands of grandchildren and great grandchildren. The main focus has been direct assessment of the hereditary risks of exposure to the atomic bombs in the offspring of the survivors (Neel et al. 1990, 1998; Otake et al. 1990; Yoshimoto et al. 1990; Little 1991, 1992). Neel et al. (1990) studied untoward pregnancy outcomes, defined as a pregnancy terminating in a child with a major congenital malformation, stillborn, or neonatal deaths occurring during the first two weeks of life. In addition, malignancies in the F_1 children with onset before the age of 20 years as well as mortality of live born infants exclusive of those resulting from malignant tumours through 26 years of age were examined. The frequency of sex-chromosomal aneuploids and the

balanced structural rearrangements of chromosomes were also studied among the offspring. Sex ratio shifts (indicative of damage to X-chromosome) were looked for among children of exposed mothers. The growth and development of the F_1 children as well as the frequency of mutations altering protein charge or function (electrophoretic variants due to point mutations) were examined. Untoward pregnancy outcomes, F_1 mortality, and cancers are multifactorial traits and their responsiveness to an increase in mutation rate (as a result of radiation exposure) will depend on the magnitude of the genetic component. Neel et al. (1990) pointed out that the genetic component is quite small. Therefore the rates of induced genetic damage underlying these traits are expected to be small, and increases will be undetectable at the low radiation doses (<200 mSv) sustained by most of the survivors. The reason for the lack of significant effects in sex-chromosomal aneuploidy is clear, as mouse studies have revealed that radiation is incapable of inducing chromosomal nondisjunction which is the principal basis for the origin of sex-chromosomal aneuploidy. Otake et al. (1990) detected that the frequency of untoward pregnancy outcomes increased unsignificantly with the combined (summed) parental dose. The authors estimated that the untoward pregnancy outcomes increased 0.00354 ($\pm$0.00343)/Sv.

Yoshimoto et al. (1990) determined cancer incidence prior to age 20 years for children born to atomic bomb survivors and a control group. Altogether, 43 malignant tumours were ascertained in the 31 150 children whose one or both parents were exposed to >10 mSv of radiation (average conjoint gonadal exposure was 430 mSv). In a control groups consisting of total of 41 066 children, 49 tumours were detected. Therefore, no increase in the malignancy of the children of exposed parents could be detected. Little (1991) did not detect an increased risk of childhood leukaemia among the children of atomic bomb survivors.

Studies of the rare electrophoretic variants of 30 proteins of blood plasma and erythrocytes and of deficiency variants of 11 erythrocyte enzymes showed no significant differences between children of exposed parents and controls (Satoh et al. 1983). In 667 404 tests of children of exposed parents and in 466 881 tests of children of control parents, only 3 probable mutations were found in both groups. Null-enzyme mutants would be expected to be induced but unlikely to be found at the dose level received by most of the A-bomb survivors.

Medical studies of the offspring of Japanese atomic bomb survivors failed to demonstrate a clear excess of genetic diseases. No statistically demonstrable adverse effects of parental radiation exposure have been found among the offspring of survivors of the atomic bombing. However, this does not mean that the gonadal mutation frequency cannot be increased. It may be increased, but so slightly that it cannot be demonstrated with the methods used in a population of this size.

2.8.3.2 Chernobyl

The catastrophic explosion of reactor 4 at the Chernobyl nuclear power station on 26 April 1986 led to the accidental release of some $5 \cdot 10^7$ Ci of assorted radionuclides, which resulted in widespread pollution, particularly in areas of the former Soviet Union. The immediate health risk to the general population was from the emission of $1 \cdot 10^7$ Ci of short-lived ^{131}I, resulting in a significant thyroid dosage whose consequence was a substantial increase in the incidence of thyroid cancer among people exposed in childhood (Kazakov et al. 1992; Likhtarev et al. 1995; Stsjazhko et al. 1995; Astakhova et al. 1998). The dose response seemed to be linear and a significant increase was seen even in children with an average dose of 50 mGy (Jacob et al. 1999). Long-term exposure has arisen mainly from environmental pollution with ^{137}Cs, which has a half-life of 30 years. ^{137}Cs caused persistent ground contamination as well as entered the food chain. The biological consequences of chronic environmental ^{137}Cs contamination for humans, including the effects of germline mutation, are unknown. A search for genetic effects associated with exposure from the Chernobyl fallout was conducted in the areas of the highest contamination (i.e., Belarus and Ukraine) and in a number of European countries. The study concentrated on congenital anomalies and other adverse reproduction outcomes.

An increased frequency of Down syndrome was reported in West Berlin in January 1987 (Sperling et al. 1994). However, a causal relationship to Chernobyl radioactivity was later excluded, because the associated cumulative dose was so low in comparison with natural background (Burkart et al. 1997). In the Lothian region of Scotland there was a significantly higher than expected incidence of Down syndroma cases in 1987 (Ramsay et al. 1991). However, this increase in Down syndroma cases could not be associated with the low levels of radioactive fallout (total deposition of ^{137}Cs of 0.1–1.0 kBq/m^2) reported in Lothian in May 1986. No evidence was seen for the incidence clustering chromosomal syndromes registered in 19 birth defect registries in Europe from January 1986 to March 1987 (De Wals et al. 1988). There was no difference in the Down syndrome frequency between the study and control groups in Finland (Harjulehto et al. 1989; Harjulehto-Mervaala et al. 1992) and even a decreasing trend was revealed in Norway (Lie et al. 199). The analysis of selected anomalies (predominantly autosomal dominant and X-linked diseases of childhood onset and Down syndrome) did not reveal any measurable germinal mutagenic effects of the Chernobyl accident in Hungary (Czeizel et al. 1991). An alleged increase in the frequency of neural tube defects and anencephaly in several small hospital-based series in Turkey (Mocan et al. 1990) was not confirmed in larger and more representative series (Mocan et al. 1992).

A statistically significant increase in spontaneous abortions with a dose of radiation was observed in Finland (Auvinen et al. 2001). However, the authors stated that the effect on spontaneous abortion should be interpreted with caution because of potential bias of confounding and because the effects of very low doses of radiation on the outcome of pregnancy are poorly known. The authors thought that the decrease in the live birth rate was probably not a biological effect of radiation, but more likely related to the public concerns of the fallout. No major effects on the outcome of pregnancy were seen in Sweden (Ericson and Källen 1994) or in Austria (Haeusler et al. 1992). A striking deviation from the linear model in early mortality rates was detected in West Germany immediately after the Chernobyl accident (Luning et al. 1989). A comprehensive review of Chernobyl studies stated that there was no consistent evidence for an increase in miscarriages, perinatal mortality, low birth weight, sex ratio shifts, or multiple births (Little 1993). However, there was evidence for an increase in induced abortions due to anxieties substantial enough to show a reduction in the total number of births.

The results of these Chernobyl studies are inconsistent, clearly illustrating that the analysis of mortality and genetic diseases among the offspring of parents exposed to radiation is not a sensitive method for monitoring radiation-induced germline mutations in humans. Therefore, minisatellite loci have been considered to offer a new experimental approach to the evaluation of germline mutation induction in humans.

2.8.3.3 Minisatellite studies

The hereditary effects of the Chernobyl accident have been studied in several exposed populations. Minisatellite analysis was chosen for study objects in these Chernobyl studies as well as in some studies of the Hiroshima and Nagasaki atomic bomb survivor cohort as it is thus far the most promising method for detecting genetic effects in humans. This is because minisatellites are non-essential for survival, have high spontaneous mutation frequencies, and are easy to detect. However, as it seems that radiation has no direct effect on minisatellite loci (effects are mediated by other systems that are themselves induced by the DNA damage) and as the biological significance of these repeats is mostly unknown, the interpretation of minisatellite results is complicated. Conflicting results obtained in the studies of radiation-induced minisatellite mutations in humans (Table 5) do not make the interpretation any easier.

The first human minisatellite studies were conducted among the offspring of atomic bomb survivors in Hiroshima and Nagasaki (Kodaira et al. 1995; Satoh et al. 1996) (Table 5). The authors sampled families in which at least one parent had been externally exposed to high doses of acute radiation. The majority of the

children studied were born more than 10 years following the exposure and derived from families in which mainly one parent had been exposed to radiation. No increase in minisatellite mutation rates was observed as minisatellite mutations were found in 1.5% of children derived from the irradiated gametes of atomic bomb survivors and in 2.0% of the control children. The later expansion of this study (Satoh and Kodaira 1996) using a multilocus minisatellite probe was also unable to show any significant difference in mutation frequencies between the children of the exposed parents and the control children despite the quite high median gonadal doses (1.9 Sv) of the exposed parents. However, because the children were born more than 10 years after the radiation exposure of their parents, these studies were unable to detect any short term effects of exposure on minisatellite mutation rate.

However, positive results were obtained by Dubrova et al. (1996, 1997) who studied minisatellite mutations in Belarus after the Chernobyl accident (Table 5). In these studies both parents of the examined children were continuously living in the contaminated area and therefore they were exposed chronically to internal and external sources of radiation. The monitoring system used revealed a nearly 2-fold increase (using all loci, 1.5-fold increase using single loci) in the mutation rates in the offspring of parents exposed to ^{137}Cs (>1 $Cikm^{-2}$) compared with unexposed control families from the United Kingdom. In the expansion of the study (1997), the authors showed that mutation rates in families with higher exposure (>20 mSv) to radiation were more elevated than in families with the lower exposure (<20 mSv), suggesting that radiation was the source of the induced mutations. This represented the first indication of a systematic genome-wide shift in the mutation rate between two different human populations, but it did not necessarily implicate environmental radiation as the inducer. The use of a control group from the United Kingdom made these data methodologically controversial and difficult to interpret. There were several possible confounding factors such as ethnicity, health status, smoking, absence of calculated dose to the parents, environmental contamination to chemical pollutants other than ^{137}Cs exposure, and inability to eliminate them (Neel 1999a).

In their further study Dubrova et al. (2002a) (Table 5) studied three-generation families in the Semipalatinsk nuclear test site in Kazakhstan. In the Semipalatinsk region of the former Soviet Union, the parents received presumably relatively high doses of radiation (>1 Sv) as a consequence of nuclear bomb tests from 1949 to 1989, including both surface and underground explosions. A significant 1.8-fold increase in minisatellite mutation rate was found in the first generation and a 1.5-fold increase in the next generation. The authors stated that the decrease in minisatellite mutation rate in the second generation was a direct result of their lower exposure. Furthermore, there was a negative

correlation between parental year of birth and mutation rates, providing evidence for decreasing mutation rate with the decay of radionuclides in Kazakhstan. There was uncertainty on the actual doses received but the increased mutation rate observed in the first generation was broadly consistent with the genetic doubling dose of 1 Sv used by the ICRP (1990) and UNSCEAR (2001) to make estimates of hereditary risks in humans after radiation exposure. However, the radiation dose estimate achieved by using FISH translocation analysis did not confirm the dose estimate of 1 Sv (Salomaa et al. 2002, see 2.8.3.6).

In their latest study Dubrova et al. (2002b) studied minisatellite mutations in Ukraine. In Ukraine, the whole-body doses from external sources for the period of time from 1986 to 2002 did not exceed 50 mSv (Dubrova et al. 2002b). These doses were well below all known estimates of the doubling dose of 1 Sv. However, the authors detected a 1.6-fold increase in minisatellite mutation rate using the same eight minisatellite loci as in their previous studies (Dubrova et al. 1997, 2002a) (Table 5). Dubrova (2003) explained their results by using other estimates of the mean doses when dose estimates ranging up to 0.5 Gy could lead to the detected 1.6-fold increase in minisatellite mutation rates. Even though the dose estimates are uncertain, the studies by Dubrova et al. (2002a, b) provide the most convincing evidence to date of hereditary mutations in humans following parental exposure to ionising radiation. However, the significance of these mutations to the health of their carriers and the following generations remains to be clarified in the future.

Unlike the studies described above, only the fathers were exposed to radiation in the studies by Livshits et al. (1999, 2001) (Table 5). They examined minisatellite mutation rates in Ukrainian Chernobyl cleanup workers exposed externally to relatively uniform levels of radiation, with a minor contribution from the intake of radionuclides. The cleanup workers received repeated small daily doses (average doses <150 mSv) of radiation over the cleanup period. Livshits and colleagues detected no increase in minisatellite mutation frequencies in children born after the Chernobyl accident to Chernobyl cleanup workers in the Ukraine compared with other Ukrainian families. The established mutation rate ratio of the exposed to control families was 0.9.

Deviating from all other human minisatellite studies as much as a 7-fold increase in minisatellite mutation rates has been documented in Chernobyl cleanup workers in the Ukraine and Israel (Weinberg et al. 1997, 1999, 2001). However, these studies have been judged unreliable because of the method used to detect the mutations (mainly RAPD-PCR, where DNA is amplified with short random-sequence PCR primers). The claimed minisatellite 'mutations' detected by the authors were not validated and had no obvious molecular basis. They may have arisen as PCR artefacts or through non-paternity or sample mix-up (Jeffreys and Dubrova 2001).

No clear evidence of an increase in the minisatellite mutation rate has been observed between pre-and post-irradiation in human sperm following radiotherapy (May et al. 2000). The study included three patients who had received hemipelvic radiotherapy. Patients received a total testicular X-ray dose between 0.38 and 0.82 Gy over a three week period. Minisatellite loci B6.7 and *CEB1* mutations were examined by using small pool PCR (SP-PCR) to analyze patient sperm samples in the pre-meiotic, meiotic, and post-meiotic cells. The authors found no evidence for mutation induction in any of the studied patients even though they were able to detect a change as low as 1.4-fold. However, the resolution in SP-PCR is conservative as very small gains or losses in repeat units may go unnoticed by this technique.

Differences in the above mentioned minisatellite studies may be explained by the obvious interpretation that there may be different biological consequences of a single acute external radiation dose received from atomic bombing compared with chronic external and internal environmental exposure received by people living in the contaminated areas and with the small, fractionated external dose to which Chernobyl cleanup workers were exposed. The evidence for mutation induction at minisatellite loci in humans remains controversial. However, all the above-mentioned data in radiation-induced minisatellites were derived from a relatively small number of families. Therefore, additional studies are needed in order to evaluate the mechanisms of mutation induction at human minisatellite loci and the possible role of radiation in it.

Although the evidence available is limited, it seems most likely that the vast majority of minisatellite mutations are genetically neutral and very few of these loci are associated with heritable diseases (Bridges 2001; UNSCEAR 2001). Those associations made for specific minisatellite loci refer largely to complex multifactorial conditions (diabetes, cancer, and spontaneous abortion) involving multiple genes and their interaction with variable environmental and lifestyle factors. According to current knowledge, the incidence of multifactorial disease in a given population is only poorly responsive to an increase in mutation rate (UNSCEAR 2001). Thus, the absolute increase in minisatellite mutations recorded by Dubrova et al. (2002a, b) will not relate directly to the incidence of genetic diseases. Minisatellites may be very sensitive to radiation-induced germline mutation in both mice and humans. In both cases, the scale of induction implies some so far unknown mechanisms whereby radiation indirectly results in mutation at tandem repeat loci. It is not possible to extrapolate these findings to other, more biologically relevant classes of mutation in the human genome, or to infer any adverse health consequences to descendants of the post-Chernobyl population. However, the current data raise questions about the mechanisms of radiation mutagenesis and consequences of environmental radiation to the future generations.

2.8.3.4 Sellafield

The hereditary effects of human radiation exposure have also been studied among nuclear power plant workers. Gardner et al. (1990) reported a statistical association between low total cumulative paternal radiation doses ($\geq$100 mSv before conception or $\geq$10 mSv in the 6 months prior to conception) and a 6-8-fold increase in the risk of leukaemia/non-Hodgkin's lymphoma in children of male workers at the Sellafield nuclear fuel reprocessing plant. This hypothesis was based on only 10 affected children and in spite of a recent study (Dickinson and Parker 2002) repeating the conclusions, the results cannot be considered independently repeated since the recent study included the same cases previously studied by Gardner et al. (1990). The median dose in the Sellafield cohort was only 29 mSv and no worker received doses greater than about 900 mSv (Parker et al. 1999; Dickinson et al. 2002). Therefore, the leukaemia of the studied children cannot be explained by the radiation exposure of the fathers and no other explanation has been found. Chance based on small number of leukaemia cases is one possible explanation as well as viral aetiology. The study by Gardner et al. (1990) prompted many other studies of nuclear power plant workers, but these studies could not confirm the possibility that radiation exposure received by a father before conception could be the cause for childhood cancer (Kinlen et al. 1993; McLaughlin et al. 1993; Doll et al. 1994; Little et al. 1995; Tawn 1995; Siffel et al. 1996; Draper et al. 1997; Neel 1999a; Wakeford 2000).

A statistical association was also reported between paternal preconception radiation exposure at the Sellafield nuclear power plant and stillbirth (Parker et al. 1999). However, the data from atomic bomb survivors (Otake et al. 1990; Little 1999) and a larger study of 13 600 nuclear industry workers in the United Kingdom (Doyle et al. 2000) did not support this finding. In addition, no correlation between preconception doses and congenital malformations was detected among children of Canadian nuclear power industry workers (Green et al. 1997).

2.8.3.5 Medical radiation exposure studies

Survivors of cancer therapy are exposed to high doses of ionising radiation before reproduction and offer a unique possibility to study the hereditary effects of radiation. The mutational analysis of this group is very complicated because radiotherapy is normally administered with chemotherapy, which itself may also be mutagenic. However, studies of the hereditary effects of radiation on patients given medical radiation are largely negative.

In spite of high radiation doses, significant genetic effects in genetic diseases attributable to parental exposure to radiation have not been found in the offspring of cancer survivors who have received radiotherapy (Byrne et al. 1998;

Sankila et al. 1998). Boice et al. (2003) conducted a large study into the hereditary effects of radiotherapy among survivors of childhood cancer. This study included >25 000 survivors whose gonadal doses were reconstructed from radiotherapy records to be >100 mSv in 46% and >1 Sv in 16% of them. In this study, the material was from the United States, 4 214 children were born to survivors of childhood cancer among whom genetic diseases were reported in 3.7% in contrast to 4.1% among 2 339 children born to sibling controls. The same study also included 1 345 children of cancer survivors and 4 225 children of sibling controls from Denmark. These children had birth defects in 6.1% and 5.0%. In addition to prior studies, this study provided reassurance that cancer radiotherapy did not carry much if any risk for hereditary diseases in offspring conceived after radiation exposure. Boice et al. (2003) concluded that although damage to the germ cells clearly occurs during radiotherapy, the complex biological processes of fertilisation, implantation, and foetal development may filter out those deleterious insults that lead to birth defects, cancer, or other serious outcomes.

In addition, hereditary effects of radiation were studied in another type of medical radiation exposure in a cohort of women who were exposed to X-rays in the therapy of scoliosis. These women were followed up for the association between radiation exposure and subsequent adverse reproductive outcomes in adulthood (Goldberg et al. 1998). Among these females, the risk of spontaneous abortions was slightly increased (OR 1.35, 95% CI 1.06-1.73), but linear dose response to radiation could not be detected. In addition, a statistically non-significant increase (OR 1.20, 95% CI 0.78-1.84) in congenital malformations in their offspring was observed.

Another type of medical radiation exposure was studied by Källen et al. (1998) who conducted a detailed analysis of the possible impact of early gonadal irradiation with β particles, γ-rays, and X-rays. They studied 19 494 infants born to 10 237 women who had been irradiated for skin haemangioma at the age of 18 months or less in Sweden. The mean ovarian dose was 0.06 Gy and the maximum, 8.55 Gy. An excess of perinatal deaths (RR 1.21, 95% CI 1.06–1.39) was evident as well as a slight increase in the rate of malformation (RR 1.08, 95% CI 1.02–1.15), but neither was related to the radiation dose received by the mother. No increase in the rate of Down syndrome or in childhood malignancies was detected.

2.8.3.6 Cytogenetic studies

The most thoroughly studied structural change that can be induced in germ cells by radiation is reciprocal translocation. It can arise in any type of germ cell and it can generally be transmitted. A significant increase in the frequency of translocation configurations has been found after spermatogonial irradiation

of human testes (Brewen and Preston 1975). However, no firm figure can be given for the risk after the low-level exposure of female germ cells, in which the induction of translocations has not been confirmed, because relevant work has not been carried out at low dose rates.

Translocation analysis using fluorescence *in situ* hybridization (FISH) chromosome painting was performed in two generations among the human population living close to the Semipalatinsk nuclear test site in Kazakhstan (Salomaa et al. 2002). Similar translocation frequencies were observed in persons living in either the Semipalatinsk area or a noncontaminated area. These findings indicate that, on average, the magnitude of exposure of this cohort has been considerably smaller than that (1–4.5 Gy) reported in the literature.

Cytogenetic studies of A-bomb exposed populations have recently been reviewed by Awa (2003). One part of these studies was a long-term cytogenetic study of the children (n = 8 322) of A-bomb survivors conducted in 1967–1984. Among these children, 19 had sex chromosome anomalies, 23 had structural chromosome rearrangements, and 1 child had trisomy. The corresponding values in the control group (n = 7 976) were 24, 27, and 9. Therefore, no statistically significant differences in the frequencies of children with abnormal karyotypes were detected in the exposed and control groups.

Cytogenetic analysis performed 4-13 years after the Chernobyl accident showed an elevated frequency of acentrics, dicentrics, rings, and chromatid exchanges among Chernobyl cleanup workers compared to the control group (Neronova et al. 2003). All these detected changes could not be attributed to radiation exposure because the frequency of acentrics was correlated with the age of the workers and the amount of chromatid exchanges increased according to the amount of smoking. However, the number of dicentrics and rings suggested a genotoxic effect of ionising radiation that was still present over 13 years after the Chernobyl accident and radiation exposure due to their cleanup activities there.

2.9 Parameters used in estimating hereditary risks of radiation

2.9.1 Background

The radiation exposure of human populations can cause an increase in the frequency of germ cell mutations which can induce genetic diseases in the descendants of those exposed. There is a clear need for methods to estimate the genetic risks associated with human radiation exposure. Both the mutant

Table 5. Minisatellite mutation studies in human populations.

Study	Exposed population	Exposed families n	Exposure	Mean dose	Children n	Control population	Control families n	Children n	Studied minisatellite loci	Mutation rate ratio (exposed / controls)
Kodaira et al. (1995), Satoh et al. (1996)	Japan A-bomb survivors	50	paternal and/or maternal	1.9 Sv	64	Japan	50	60	*CEB1;λTM-18; ChdTC-15; pλg3; λMS-1*	0.8
Satoh & Kodaira (1996)	Japan A-bomb survivors	50	paternal and/or maternal	1.9 Sv	64	Japan	50	60	*33.15*	0.9
Dubrova et al. (1996, 1997)	Belarus	125	paternal and maternal	>1 Cikm^{-2} surface	125	United Kingdom	109	109	*B6.7; CEB1, 15, 25, 36; MS 1, 31, 32*	1.5 (pat.) 2.0 (mat.)
Dubrova et al. (2002a)	Kazakhstan	40 (3 gen.)	paternal and maternal	P_0: >1 Sv? F_1: less than P_0	total 361	Kazakhstan	28 (3 gen.)	total 251	*B6.7; CEB1, 15, 25, 36; MS 1, 31, 32*	1.8 (P_0) 1.5 (F_1)
Dubrova et al. (2002b)	Ukraine	171	paternal and maternal	<50 mSv or even 0.5 Gy?	240	Ukraine	54	98	*B6.7; CEB1, 15, 25, 36; MS 1, 31, 32*	1.6 (pat.) 1.0 (mat)
Livshits et al. (1999, 2001)	Ukraine Chernobyl cleanup workers	161	paternal	<150 mSv	183	Ukraine	163	163	*B6.7; CEB1, 15, 25, 36, 42, 72*	0.9 (pat.)
The present study	Estonia Chernobyl cleanup workers	147	paternal	109 mSv	155	the same families	147	148	*B6.7; CEB1, 15, 25, 36; MS 1, 31, 32*	1.2 (pat.) 1.2 (mat.)

genetic risks associated with human radiation exposure. Both the mutant frequency and the molecular spectrum of gene mutations need to be considered in the assessment of radiation-induced genetic risk. Data on mouse germ cell mutation rate following spermatogonial exposure to high dose rate irradiation have been presented as the most relevant experimental results upon which to extrapolate the expected genetic risk in humans (UNSCEAR 2001). However, caution must be exercised when extrapolating from the animal studies to the human situation, as the mouse strain used and even different colonies of the same mouse strain in different laboratories have their influence on the observed effects. High radiation doses have been used in mice studies but the question of low-dose extrapolation is of utmost importance when estimating the genetic risk from radiation in humans. There has been a general international consensus that it would be prudent to assume linearity at low doses (<0.1–0.2 Gy) in the absence of convincing data to the contrary. Most mutations, such as deletions, that are conventionally considered to constitute a genetic risk arise from complex DNA lesions that are produced linearly with dose at low doses (Goodhead 1994). However, if mutations were to arise from indirect processes (e.g., minisatellite mutation, genomic instability, bystander effect), then the biophysical argument would collapse, since the nature of the lesions that trigger such responses is not known. Thus far it is unknow if these phenomena reflect lesions in DNA or the relationship between indirect responses and initial damage. The following parameters are traditionally used in the assessment of radiation-induced hereditary risks in humans.

2.9.2 Doubling dose

Radiation-induced hereditary effects are often measured by using the indirect or doubling dose method (see 2.10.3). Doubling dose (DD) is the gonadal radiation dose required to produce as many mutations as those occurring spontaneously in a generation, i.e., doubling (= 2-fold increase) of the mutation rate of the control population. It is estimated as a ratio of the average spontaneous mutation rate of a given set of genes relative to the average rate of induction of mutations by radiation in the same set of genes (Sankaranarayanan and Chakraborty 2000a):

$$DD = \frac{m}{\Delta m}, \qquad (4)$$

where: m = average spontaneous mutation rate
Δm = average increase in the mutation rate/unit dose.

Genes that mutate at high rates spontaneously are assumed to behave similarly after irradiation. However, this assumption is open to criticism. A high spontaneous rate depends, among other factors, on the size and sequence of the genes in question and of the types of mutational mechanisms involved. A high induction rate is more dependent on whether a random change in the gene can give rise to the phenotypes being observed.

$$\text{Risk / unit dose} = P \cdot \frac{1}{DD}\ , \qquad (5)$$

where: P = baseline (equilibrium) incidence
1/DD = relative mutation risk/unit dose.

The population genetic theory that underlies the equation (5) is the equilibrium theory (see 2.3.3). The P in the equation (5) represents the equilibrium incidence, which is directly proportional to the mutation rate. If the mutation rate is increased as a result of radiation in every generation, the prediction is that the population will attain a new balance (over a number of generations) between mutation and selection.

It would have been ideal to base DD estimates on human spontaneous and induced rates of mutations. However, as human data on radiation-induced germ cell mutations is still lacking, a less ideal approach of using human data on spontaneous mutation rates and mouse data on induced rates have to be used. Several mouse mutation systems have been used for this purpose (Table 6).

The doubling dose (DD) of 1 Gy based on mouse data has been used in humans at least during the past 20 years. Based on the results of mice studies, Selby (1998 a) suggested that it would be reasonable to raise the doubling dose from 1 Gy to 5 Gy for low-dose-rate exposure to X- and γ-irradiation. Doing so would reduce the risk estimates made by the DD method fivefold. An even higher estimate of the DD as the computer stimulation of control experiments involving 57.4 million offspring in mice showed that the total frequency of spontaneous mutations/generation is much higher than thought because of gonadal mosaicism. Therefore, the estimate of the DD could be increased to as much as 7.7 Gy (Selby 1998b). However, Russell and Russell (1959) have pointed out the fallacy of comparing DD estimates for mutations that were induced at different stages of gametogenesis, in different sexes, or under different dose rates. It has also to be born in mind that spontaneous mutation rates in humans and mice are dissimilar. Unlike in mouse, the mutation rate in humans differs between the two sexes (higher in males than in females) and increases with paternal age. Germinal mosaic mutations in mice, which result in clusters of identical mutations in the following generations, compose another source of uncertainty.

Table 6. Estimates of the gonadal doubling dose for acute radiation of spermatogonia in mice.

System	Spontaneous mutation rate	Total tested[1]	Exposure (Gy)	Doubling dose (Gy) (95% CI)[2]
Russell 7-locus[3-4]	$7.95 \cdot 10^{-6}$	1 051 869	3, 6, 6.7	0.34 (0.22-0.50)
Dominant visibles[4]	$8.11 \cdot 10^{-6}$	225 017	6, 12	0.17 (0.00-0.59)
Dominant cataract[5] (30 loci)	$7.38 \cdot 10^{-7}$	84 750	6	0.38 (-0.14-3.75)
Enzyme activity[6] (12 loci)	$2.85 \cdot 10^{-6}$	36 422	6	0.44 (-0.09-2.68)
Skeletal malformations[4]	$2.88 \cdot 10^{-4}$	2 493	6	0.27 (-0.07-1.67)
Semi-sterility[4]	$1.04 \cdot 10^{-3}$	2 124	12	0.31 (0.03-0.95)
Minisatellites[7]	$5.56 \cdot 10^{-2}$	252	0.5, 1	0.33 (0.06-0.75)
Mean for 7 systems	-	-	-	**0.35** (0.20-0.95)

[1]Including offspring from the control and irradiated parents, [2]Confidence limits computed from the Poisson distribution, [3]Russell and Kelly 1982, [4]Lüning and Searle 1971, [5]Favor 1989, [6]Pretsch et al. 1994, [7]Dubrova et al. 1998a, b.

From the Hiroshima and Nagasaki data it has been estimated that the doubling dose (DD) in humans is 2 Sv for acute exposure and about 4 Sv for chronic exposure (zygotic rather than gametic DD) (Neel 1998, 1999b). The data did not exclude estimates of the zygotic DD of acute radiation as high as 3, 4, or 5 Sv equivalents. Using human data on spontaneous mutation rates (estimate of $2.95 \pm 0.64 \cdot 10^{-6}$/locus/generation) and mice data on induced rates (estimate of $3.6 \pm 0.10 \cdot 10^{-6}$/locus/Gy), UNSCEAR (2001) yields a DD estimate of 0.82 Gy. Using the variance of 0.0834 and standard error of 0.29, the currently revised DD of 0.82 ± 0.29 Gy is not significantly different from 1 Gy. Therefore, UNSCEAR (2001) concluded that the round figure of 1 Gy for chronic, low-dose, low-LET radiation exposure can also be used in risk estimation for for DD for both sexes in the future.

2.9.3 Mutation component

The mutation component (MC) is a measure of the proportion of the impact of a genetic condition that is attributable to recurrent mutation (Crow and Denniston 1981). MC predicts the extent to which the frequencies of a given class of diseases increase as a result of, e.g., radiation exposure, i.e., MC is the fraction of the disease incidence that is proportional to mutation rate.

The formal definition of the mutation component (MC) is:

$$MC = \frac{\Delta P}{P} / \frac{\Delta m}{m}, \quad (6)$$

where: P = disease incidence before radiation
(baseline incidence)
ΔP = change in P due to new induced mutations
m = spontaneous mutation rate
Δm = change in mutation rate as a result of radiation.

In the first generation the empirical mouse data on induced developmental abnormalities can be used. For the second and later post-radiation generations, knowledge of MC (which is dictated by the magnitude of the selection coefficient, s) is required:

$$\text{Risk / unit dose} = P \cdot \frac{1}{DD} \cdot MC, \quad (7)$$

where: P = baseline incidence
1/DD = relative mutation risk/unit dose
MC = mutation component.

The mutation rate of a hypothetical autosomal dominant mutation in a population under equilibrium becomes twice that of the spontaneous rate as a result of radiation exposure. This doubling of the mutation rate occurs in either one generation or generation after generation. For a one-time (burst, subscript b) increase in the mutation rate in any generation is (Chakraborty et al. 1998):

$$MC_b(t) = s(1 - s)^{t-1}, \quad (8)$$

where: t = time
s = selection coefficient.

For a permanent (subscript p) increase in the mutation rate (Chakraborty et al. 1998):

$$MC_p(t) = 1 - (1 - s)^t, \quad (9)$$

where: t = time
s = selection coefficient.

A one-time or a permanent increase in the mutation rate has the same impact in the first generation following the increase, namely, $MC_b = MC_p = s$. With no irradiation in subsequent generations, the mutation component will gradually decay to zero at a rate of (1-s)/generation. With a permanent increase in the mutation rate, the mutation component will continue to increase in subsequent generations to attain a value of 1 at the new equilibrium.

The mutation component (MC) can be applied to Mendelian diseases (Chakraborty et al. 1998) and multifactorial diseases (Denniston et al. 1998). An estimate of the average selection coefficient, s, for autosomal dominant and X-linked diseases is of the order of $\sim$ 0.3. It is a value that defines the first generation MC. Therefore, the value of MC = 0.3 can be used for computing the risk for autosomal dominant and X-linked diseases for the first post-irradiation generation. In autosomal recessive diseases the mutation component is close to zero in the first few generations. For multifactorial diseases as a whole, MC can vary between 0.05 and 0.5; MC = 0.02 can be used for the first several post-irradiation generations when there is radiation exposure in every generation. For all induced developmental defects considered overall, s may be in the range of 0.2 to 0.5 for the second post-irradiation generation (meaning that 20% to 50% of the abnormal progeny may transmit the damage to the second generation).

2.9.4 Potential recoverability correction factor

The potential recoverability correction factor (PRCF) (Sankaranarayanan and Chakraborty 2000b) defines the recoverability of radiation-induced mutations in human live births. PRCFs are disease-class specific. As PRCFs are based on the known difference (in nature, type, and mechanisms) between spontaneous disease-causing mutations in humans and radiation-induced mutations in mice, they bridge the gap between radiation-induced mutations determined in mouse studies and the risk of inducible genetic diseases in human live births. The criteria for assessing the potential recoverability of radiation-induced mutations were developed on the basis of recovered mutations in mouse experiments. They were applied to human genes of interest from the disease point of view. Recoverability of radiation-induced deletions in live births in humans seems to be dependent on whether the loss of gene/genomic region is compatible with viability in heterozygotes. Human gene size, location, normal function, known mutational mechanisms, the spectrum of naturally occurring mutations, gene-richness of the region, etc. are considered in the assessment.

Sankaranarayanan and Chakraborty (2000b) state that the potential recoverability of an induced deletion of a gene/genome region can be judged as potentially recoverable if rearrangements (e.g., translocations or inversions)

are known to occur with breakpoints in the gene/genomic region of interest and produce the disease phenotype. These gene/genomic regions are non-essential for viability of the heterozygotes and large, whole gene and multi-locus deletions are known to occur in these regions. Potential recoverability can be judged as uncertain when the genomic context information of the gene is insufficient and large intragenic and multilocus deletions of the gene are rare (presumably because of the lethality of heterozygotes). Uncertain potential recoverability is also attached to large genes coding for a structural protein and genes where known genetic changes are missense and nonsense mutations or small intragenic deletions or insertions (causing frameshifts and predicted to result in truncated proteins). Potential recoverability can be judged unlikely when mutations in the gene/genomic region are specific point mutations or specific tandem duplications causing gain-of-function. Recoverabilited gene/genome regions where mutations are point mutations or small specific intragenic deletions causing loss-of-function due to dominant negative mechanisms are also unlikely. In addition, when a gene is in expansion of trinucleotide repeats its potential recoverability is unlikely.

The PRCF varies between 0.15 and 0.30 for autosomal dominant and X-linked diseases. There is no need to estimate the PRCF for autosomal recessive disorders, since the mutation component is close to zero. For multifactorial diseases, the PRCF range of 0.02 to 0.09 is used. For congenital abnormalities, PRCF calculations are unnecessary, since the available empirical mouse data on developmental abnormalities can be used to obtain an approximate estimate of risk without recourse to the DD method. The range for PRCF reflects biological and not statistical uncertainties.

2.10 Methods for hereditary risk estimation

2.10.1 Background

The aim of hereditary risk estimation is to predict the impact of a small radiation dose on disease incidence in the population. It is assumed that any small dose of radiation causes an increase in the mutation rate. Two extrapolation procedures, based on experimental data from mice, are available to estimate the genetic risk in humans. They are termed the direct and indirect or doubling dose method.

2.10.2 Direct method

In estimating hereditary risk it is assumed that the 7-loci in mice constitute a suitable basis for extrapolation to genetic disease in humans, and that

mutations are increasing linearly with dose (at least at low doses) within the gene. Recent evidence on the mutability of repeat sequences suggests that both these assumptions are wrong and consequently, the direct method has now been abandoned (UNSCEAR 2001). However, the data on skeletal abnormalities and cataracts used with the direct method (along with those on congenital abnormalities diagnosed in utero) now provide the basis for estimating the risk of developmental abnormalities as a whole. It is no longer considered necessary to make separate estimates of the risk of chromosomal diseases since these are already assumed to be included with developmental abnormalities.

2.10.3 The indirect or doubling dose method

The doubling dose (DD) method estimates the hereditary risk in terms of the additional number of cases of genetic diseases due to radiation exposure relative to the prevalence of those occurring naturally in the population. The DD is estimated in mice and the spontaneous mutation rate is estimated in humans. Such an extrapolation from mouse data to the risk of genetic diseases is valid only if the average rates of inducible mutations in human genes of interest and the average rates of induced mutations in mice are similar (Sankaranarayanan and Chakraborty 2000b). The advantages of this method include the fact that estimates of spontaneous mutation rates in humans (usually presented as sex-averaged rates) automatically include sex differences and paternal age-effects. Moreover, if clusters of mutations have occurred, they are included in the mutation rate calculations as all human mutations are counted irrespective of whether they are part of a cluster or not. The DD was found to be four times higher in the dominant cataract test than the 7-locus test in mice. These results indicate that to extrapolate to genetic risks in humans using the DD method, the extrapolation must be based on the experimental mutation rate results for the same genetic endpoint (Sankaranarayanan and Chakraborty 2000b).

Since the risk due to radiation is conventionally expressed as the expected number of cases of genetic disease (over and above the baseline incidence) for autosomal dominant diseases, the risk is estimated as:

$$\text{Risk / unit dose} = P \cdot \frac{1}{DD} \cdot MC \cdot PRCF\,, \qquad (10)$$

where: P = baseline frequency (incidence) of the genetic disease under consideration
DD = doubling dose (see 2.9.2.)
1/DD = relative mutation risk/unit dose (see 2.9.2)
MC = mutation component (see 2.9.3)
PRCF = potential recoverability correction factor (see 2.9.4).

From epidemiological studies, P can be estimated to be 2.4% for Mendelian diseases, 0.4% for chromosomal diseases, and 71% for multifactorial disease (Czeizel and Sankaranarayanan 1984; Czeizel et al. 1988). For autosomal dominant diseases, if there is an x% increase in the mutation rate, it will be reflected as an x% increase in disease at the new equilibrium. The population genetic theory of equilibrium (see 2.3.3) between mutation and selection underlies the use of the DD method. The equilibrium theory predicts that under conditions of a permanent increase in mutation rate, the MC will reach a value of 1 at the new equilibrium for both Mendelian and multifactorial diseases. For all classes of genetic diseases, the time it takes to reach the new equilibrium is critically dependent on the magnitude of selection. For congenital abnormalities, an estimate of risk at the new equilibrium is very difficult to obtain in view of the fact that a progressive increase in genetic damage in the genome over time may dramatically increase the magnitude of selection, such that individuals affected with these developmental abnormalities may not survive to reproductive age to transmit the damage. Radiation risk estimates calculated using the indirect (DD) method and the equation (10) are seen in Table 7. These risk estimates are valid if the whole radiation dose is genetically significant and is applicable to a reproductive population. The genetically significant dose for the whole population will be markedly lower than the total dose received over a lifetime, as damage in the germ cells of individuals with no offspring produce no risk. In the future, computation modelling will be needed to estimate radiation risks in heterogeneous human populations.

Table 7. Radiation risk estimates using the indirect (doubling dose) method [based on the Eq. (10), Sankaranarayanan and Chakraborty 2000c and UNSCEAR 2001].

Disease	Risk/Gy of parental irradiation (cases/million live born)[4]				
	Radiation exposure in one generation		Radiation exposure in every generation		
	1st gen. progeny	2nd gen. progeny	1st gen. progeny	2nd gen. progeny	at new equilibrium
AD[1]	≈ 750-1 500[5]	≈ 500-1 000[6]	≈ 750-1 500[5]	≈ 1 300-2 500[7]	≈ 2 500-5 000[8]
AR[2]	0[9]	0[9]	0[9]	0[9]	≈ 1 100-2 200[10]
X-linked	≈ 750-1 500[5]	≈ 500-1 000[6]	≈ 750-1 500[5]	≈ 1 300-2 500[7]	≈ 2 500-5 000[8]
mf [3]	≈ 250-1 200[11]	≈ 250-1 200[11]	≈ 250-1 200[11]	≈ 250-1 200[11]	≈ 13 000-58 500[12]
dev.abn.	2 000[13]	400-1 000[14]	2 000[13]	2 400-3 000[15]	
cong. abn.	0[16]	0[16]	0[16]	0[16]	
total %[17]	**0.41-0.64**			**0.53-0.91**[18]	

[1]Autosomal dominant disease, [2]Autosomal recessive disease, [3]Multifactorial disease, [4]Risk/Gy = P · (1/DD) · MC · PRCF (eq. 10) (at new equilibrium additional cases/million), [5](16 500 · 10^{-6}) · 1 · 0.3 · (0.15-0.30), [6](16 500 · 10^{-6}) · 1 · (0.3 · 0.7) · (0.15-0.30), [7](16 500 · 10^{-6}) · 1 · [1 - (1 - 0.3)^2] · (0.15-0.30), [8](16 500 · 10^{-6}) · 1 · 1 · (0.15-0.30), [9]MC ≈ 0, [10](7 500 · 10^{-6}) · 1 · 1 · (0.15-0.30) (if the PRCF estimated for autosomal dominant and X-linked diseases are also assumed to be applicable to recessive diseases), [11](650 000 · 10^{-6}) · 1 · 0.02 · (0.02-0.09), [12](650 000 · 10^{-6}) · 1 · 1 · (0.02-0.09), [13]based on mouse data on skeletal abnormalities, cataracts and congenital abnormalities scored *in utero*, [14]s = 0.2-0.5, [15][(0.2-0.5) · 2 000] + 2 000, [16]for congenital abnormalities, complete selection has been assumed (i.e., none is transmitted, s = 1) and consequently all cases are new, [17]of the baseline frequency of these diseases (738 000/million) in the population, [18]includes the risk to the first generation as well.

3 Aims of the present study

The general purpose of the present study was to evaluate the applicability of the minisatellite mutation screening method for assessing radiation-induced hereditary risks in humans.

The specific aims of the study were:

1. To test the usability of PCR-based minisatellite mutation screening methods in family studies.

2. To determine the rate of heritable minisatellite mutations in the radiation exposed population of Estonian Chernobyl cleanup workers.

3. To examine if the minisatellite mutation rate increased due to father's exposure to radiation while working at Chernobyl.

4. To determine if there is a correlation between the minisatellite mutation rate and the father's radiation dose.

5. To test if the paternal age effect can be seen in the minisatellite mutation rate.

6. To determine the effect of a father's spermatogonial stage during the radiation exposure in the minisatellite mutation rate

7. To determine the applicability of the linear dose response to this material.

8. To examine if the doubling dose can be established based on the material used.

9. To compare paternal and maternal minisatellite mutation rates.

10. To determine minisatellite mutation rates in other Estonian control families.

4 Materials and methods

4.1 Subjects

The reactor accident at Chernobyl at the 26th April 1986 necessitated a massive environmental cleanup that involved over 600 000 workers from all 15 republics of the former Soviet Union. The cohort of Estonian cleanup workers consisted of 4 832 men. They participated, mostly at ages 20–39 years, in cleanup activities at Chernobyl over a median period of three months between 1986 (60.4% of workers) and 1991. Their mean radiation dose was estimated to be 109 mSv (SE ± 63 mSv). Less than 1.4% of the cohort received more than 250 mSv. The estimate of the men's individual radiation dose was obtained from the database of the Estonian Study of Chernobyl Cleanup Workers (Inskip et al. 1997; Rahu et al. 1997; Tekkel et al. 1997). This dose was a physical dose based primarily (70% of the men) on thermoluminescence dosimeter (TLD) readings, which are an estimate of the external whole-body dose from gamma irradiation. The estimated radiation dose was considered to represent external radiation exposure, reflecting the gonadal dose. Studies using biological dosimetry (GPA assay, chromosome analysis, FISH) yielded comparable estimates of average dose (Salassidis et al. 1994; Granath et al. 1996; Bigbee et al. 1997; Littlefield et al. 1998).

4.2 Samples and dna extraction

Within the cohort, men with children born both prior to and after the accident were identified from questionnaires (Tekkel et al. 1997) and those with a child conceived within 33 months of return from Chernobyl were selected for the study (n = 208). Of them, 192 gave consent and blood samples were collected from 742 persons from 192 Estonian families (192 fathers, 187 mothers, 198 post-Chernobyl children, 165 pre-Chernobyl children). Approximately 20 ml of blood from each donor was collected into sterile Vacutainers and maintained at ambient temperature until delivery to STUK within 24 hours after venipuncture. The blood samples were coded by family study number with the code F for father, M for mother and C1, C2, or C3 arbitrarily (not in order of age) given for children, thus blinding later analyses (analyses were conducted without the knowledge of whether the children belonged to the pre- or post-Chernobyl group). Genomic DNA was extracted from freshly isolated blood lymphocytes using QIAamp Tissue Kit (QIAGEN, Valencia, USA) according to the manufacturer's instructions. The DNAs were stored in 1 x TE buffer in -20°C.

After excluding incomplete families and families with non-paternity using PCR-based methods (see 5.1.), DNA samples from 597 persons from 147 families of Estonian Chernobyl cleanup workers composed the final study material. The "exposed" children (n = 155) were conceived within 33 months of the father's return from Chernobyl. Their siblings (n = 148) born prior to the accident formed the reference group. This design allowed us to use an optimal reference group, with matching for inter-individual variability in, e.g., genetic factors. Thus, we were able to maximise the comparability of genetic and other factors. To have an estimate of the time of conception, and thus of the stage of the spermatogenesis during irradiation, the gestation age of the children at birth was assumed to be 40 weeks.

Blood samples were also collected from 144 persons from 48 Estonian families (father, mother, and child) where the father had not been exposed to radiation (neither at Chernobyl nor occupationally). These blood samples were sent to STUK, coded, and handled identically to other blood samples. This additional control group was collected to provide an external reference rate for the assessment of generalizability (selection). After excluding families with non-paternity, 44 families composed the final additional control group.

4.3 Polymerase chain reaction-based analyses

The biological paternity of all children was ascertained by the polymerase chain reaction (PCR)-based analysis of five minisatellite loci. Minisatellite loci *APOB, HRAS, MCOB19, MCT118,* and *YNZ-22* were chosen for paternity testing because of their reliability due to their relative low spontaneous mutation frequency. Some basic features of the used loci are seen in Table 8.

Table 8. Minisatellite loci studied by PCR-based analyses.

Minisatellite locus	Locus symbol	Map location	Repeat unit (bp)	Number of alleles	Allele size	Heterozygosity (%)
APOB	APOB	2p24-p23	13-15	25	500-1 000	75-77
HRAS	HRAS	11p15.5	28	30	1 000-3 000	72
MCOB19	D19S20	19p13.3	33	18	200-4 000	70-79
MCT118	D1S80	1p36-p35	16	21	430-750	79
YNZ-22	D17S5	17p13.3	70	14	170-1 070	85-88

Following flanking primers were used for PCR-based minisatellite analyses:

APOB F	5´-ATGGAAACGGAGAAATTATG-3´
APOB R	5´-CCTTCTCACTTGGCAAATAC-3´
HRAS F	5´-TTGGGGGAGAGCTAGCAGGG-3´
HRAS R	5´-CCTCCTGCACAGGGTCACCT-3´
MCOB19 F	5´-AGATGCCTTACTGAGAAGCGGGAG-3´
MCOB19 R	5´-TTGTCCACGGGTATTTATCATACGG-3´
MCT118 F	5´-GAAACTGGCCTCCAAACACTGCCCGCCG-3´
MCT118 R	5´-GTCTTGTTGGAGATGCACGTGCCCCTTGC-3´
YNZ-22 F	5´-TCGAAGAGTGAAGTGCACAGGAGGGCAAG-3´
YNZ-22 R	5´-GGGTCTGCTGTGCCCACAGTCTTTATTCT-3´

In each primer pair the forward primer was labelled with a fluorescent dye, thus the amplification products of *APOB* and *YNZ-22* carried green (HEX), *HRAS* and *MCOB19* blue (6-FAM), and *MCT118* yellow (TAMRA) fluorescence. All the used primers have been published earlier (*APOB*: Boerwinkle et al. 1989; *HRAS*: Ugozzoli et al. 1991; *MCOB19, MCT118* and *YNZ-22*: Tully et al. 1993).

PCR-conditions (Table 9) and programs were optimized for each amplified minisatellite locus in a total volume of 50 μl. The initial denaturation at +94°C for 5 minutes and the final cooling to +4°C was included in each program. *APOB* was amplified using a diphasic PCR-program at +94°C for 30 seconds for denaturation and +58°C for 6 minutes for annealing and extension, which was repeated for 26 cycles. The final extension at +58°C for 7 minutes completed the amplified *APOB* fragments. PCR-programs consisting of three phases were used for all other minisatellite loci. The PCR-program for *HRAS* contained 25 cycles repeating denaturation at +94°C for 20 seconds, annealing at +64°C for 30 seconds, and extension at +68°C for 3 minutes. The final extension at +68°C for 7 minutes finished the program. A polymerase designed for amplifying longer alleles (Table 9) was used in amplifying *HRAS* because large alleles were frequent in this minisatellite locus. The *MCT118* locus was amplified using a PCR-program at +94°C for 30 seconds, +62°C for 30 seconds, and +72°C for 1.5 minutes repeated 27 times for denaturation, annealing, and extension. This PCR-program was finalised with the extension at +72°C for 7 minutes. The same PCR-program was used for amplifying *MCOB19* and *YNZ-22* minisatellite loci. This program consisted of denaturation at +94°C for 30 seconds, annealing at +62°C for 30 seconds, and extension at +72°C for 3 minutes. Denaturation, annealing, and extension were repeated for 30 cycles. The final extension at +72°C for 7 minutes completed the PCR-program.

The result of the PCR-amplification was checked by electrophoresing a 10 μl aliquot of each PCR-product through 0.8% (*HRAS*) or 1.8% (all other loci) (w/v) agarose gels (Agarose NA, Pharmacia, Sweden). The gels were run with 80 V for 2 hours in 1 x TBE buffer (89 mM Tris-borate, pH 8.3, 2 mM EDTA). When minisatellite loci *MCOB19* and *YNZ-22* were studied, some families had large alleles, which were amplified inefficiently. Therefore, PCR-based minisatellite analysis was repeated in these families using other PCR-conditions (Table 9) optimised for especially long alleles using a polymerase designed specifically for amplifying long PCR-fragments (DyNAzyme EXT DNA polymerase, Finnzymes, Espoo, Finland). These especially long alleles were amplified by 30 cycles at +94°C for 20 seconds for denaturation, +62°C for 30 seconds for annealing, and +68°C for 3 minutes for extending. A final extension at +68°C for 7 minutes completed the extension of the amplified fragments. The PCR machine was programmed to keep the samples at 4°C for an unspecified time (∞) after the PCR was completed.

Table 9. Optimized PCR-conditions used in minisatellite analyses.

Locus	DNA (ng)	Primers (μM)	dNTPs (mM)	$MgCl_2$ (mM)	KCl (mM)	Triton X–100 (%)	Tris-HCl (pH 8.8) (mM)	$(NH_4)_2SO_4$ (mM)	Poly-merase (U)
APOB	100	1.5	0.20	1.50	50.0	0.1	10.0	-	3.0[1]
HRAS	250	0.4	0.36	1.75	50.0	0.1	-	15.0	2.0[2]
MCOB19	250	0.2	0.20	1.50	50.0	0.1	10.0	-	2.0[1]
MCOB19[3]	250	0.2	0.36	1.75	50.0	0.1	-	15.0	2.0[2]
MCT118	100	0.2	0.20	1.50	50.0	0.1	10.0	-	2.0[1]
YNZ-22	100	0.2	0.20	1.50	50.0	0.1	10.0	-	2.0[1]
YNZ-22[3]	100	0.4	0.36	1.75	50.0	0.1	-	15.0	2.0[2]

[1]DyNAzyme™ II DNA polymerase (Finnzymes, Espoo, Finland), [2]DyNAzyme EXT DNA polymerase (Finnzymes, Espoo, Finland), [3]Other PCR-conditions used for especially long alleles.

Small samples (0.5-2 μl) of successfully amplified PRC-products from complete families were electrophoresed through 4.5% *(HRAS)* or 6% (all other loci) native polyacrylamide (PAGE) (Acryla-mide/Bis 29:1) (Bio-Rad, Hercules, USA) gels. Native 6% PAGEs were run with 2500 V and 13 W (~800 V) for 13 hours using automated ABI PRISM 377 DNA sequencer (Applied Biosystems, Fos-ter City, USA). For 4.5% PAGEs 2500 V and 14 W (~800 V) were used for a 12-hour electrophoresis. Amplified PCR-fragments from minisatellite loci

MCOB19 and *YNZ-22* of one person were run simultaneously on the same lane; all other loci were studied individually. The fluorescent bands obtained for each family were analysed with the GeneScan program (Perkin-Elmer, Boston, USA). Mutations in children were seen as fragments whose size differed from the sizes of the parental fragments.

4.4 Southern blot-based analyses

For the Southern blot–based analysis, 4 μg samples of genomic DNA were digested to completion with *AluI* (Promega, Madison, WI, USA), using reaction conditions recommended by the supplier. The samples were electrophoretically separated through 25-cm long 0.8% (w/v) agarose gels (Agarose NA, Pharmacia, Sweden) for 24 hours in 1 x TBE buffer (89 mM Tris-borate, pH 8.3, 2 mM EDTA). Each gel included three lanes carrying λ/*HindIII* fragments (Promega, Madison, WI, USA) as molecular weight markers. Separated DNA fragments were transferred to a nylon membrane (Hybond N+, Amersham, Buckinghamshire, UK).

Minisatellite loci *B6.7, CEB1, CEB15, CEB25, CEB36, MS1, MS31*, and *MS32* were chosen for Southern blot–based minisatellite mutation analysis due to their relatively high spontaneous mutation rate (Jeffreys et al. 1988, 1991b; Vergnaud et al. 1991; Olaisen et al. 1993; Tamaki et al. 1999; Vergnaud and Denoeud 2000). Out of these eight hypervariable single-locus probes (Table 10), probe CEB1 for Southern blotting was produced from a PCR fragment. Used primers were (Buard and Vergnaud 1994):

CEB 1F 5´-GGTCTAGAGCTCTGCTGAGTCAGAGTCAGCCAG-3´
CEB 1R 5´-AAACTGTAATCTGGAGTTGGTCTGGCGATC-3´.

During the PCR, 500 ng of template DNA was amplified in a PCR mix containing 0.4 μM of each primer, 0.36 mM dNTP, 1.75 mM MgCl2, 2% DMSO, and 100 μl of Dynawax (Finnzymes, Espoo, Finland). Above the Dynawax was added 2 U of Dynazyme EXT (Finnzymes, Espoo, Finland) DNA polymerase. The total volume of the PCR was 10 μl. Amplification of *CEB1* alleles was initiated with denaturation at 94°C for 2 minutes. Amplification was continued with 10 cycles at +96°C for 10 seconds for denaturation, +62°C for 30 seconds for annealing, and +68°C for 6 minutes for extending. Following that were 20 identical cycles, with the exception that the extending temperature (+68°C) lasted 20 seconds longer in each cycle. A final extension at +68°C for 7 minutes completed the extension of the amplified fragments. The PCR machine was programmed to keep the samples at 4°C for an unspecified time (∞) after the PCR was completed. The amplified PCR fragment was cloned into vector pCR 2.1. The host bacterium used

was INValphaF´ with kanamycin selection. The cloning product was digested with *EcoRI*. This digestion gave a 1.2 kb restriction fragment that was used as a CEB1 probe in Southern blotting.

Probes CEB15, CEB25, CEB36, MS32, and B6.7 were received as gifts (see acknowledgements). These probes were transformed and cloned using a TOPO™TA cloning kit (Invitrogen, Carlsbad, USA) under the conditions specified in Table 11. Probes MS1 and MS31 were commercially purchased (Cellmark Diagnostics, Oxfordshire, UK).

Table 10. Minisatellite loci studied by Southern blot-based analyses.

Mini-satellite locus	Locus symbol	Map location	Repeat unit (bp)	No. of alleles	Allele size (bp)	Spont. male mut. freq.* (%/gam.)	Spont. female mut. freq.* (%/gam.)	Hetero-tsygosity (%)
B6.7		20q13.33	34	2 000	204 - 17 000	7.6	3.9	99.9
CEB1	D2S90	2q37.3	39	109	500 - 12 000	25.0	0.4	93.0
CEB15	D1S172	1p36.33	18	116	500 - 11 000	2.4	0	94.0
CEB25	D10S180	10q26.3	52	109	600 - 16 000	3.2	1.9	97.0
CEB36	D10S473	10q26.3	42	108	500 - 7 000	1.8	1.8	97.0
MS1	D1S7	1p33-p35	9	132	540 - 20 000	5.4	5.4	99.0
MS31	D7S21	7p22-pter	20	124	2 000 - 13 000	1.0	1.0	98.0
MS32	D1S8	1q42-q43	29	122	1 000 - 20 000	0.8	0.8	97.5

*Spontaneous minisatellite mutation frequencies vary from individual to individual from <0.05% to these figures depending on size of the minisatellite allele of the individual

Table 11. Conditions used for cloning minisatellite probes.

Probe	Vector	Host bacterium	LB bacteria growing medium + selection antibiotic (50 µg/ml)	Restriction enzyme(s)	Length of the digestion fragment (kb)
B6.7	cosmid pWE15	TOP10	ampicillin	*AluI + HinfI*	2.7
CEB1	pCR 2.1	INVαF'	kanamycin	*EcoRI*	1.2
CEB15	pUC 13	TOP10	ampicillin	*HinfI*	3.8
CEB25	cosmid pWE15	TOP10	kanamycin	*AluI + HaeIII*	1.9
CEB36	pUC13	TOP10	ampicillin	*AluI + HaeIII + HinfI*	2.5
MS32	pUC13	TOP10	ampicillin	*BamHI*	5.6

The samples were hybridised to fluorescein-11-dUTP random primed labelled probes. The probes were attached to an anti-fluorescein antibody conjugate including alkaline phosphatase (all CEB probes, MS32, and B6.7) or to commercial, alkaline phosphatase labelled single-stranded oligonucleotide NICE™ (probes MS1 and MS31, Cellmark Diagnostics, Oxfordshire, UK). Detection was achieved via alkaline phosphatase catalysing the chemiluminescent reaction. The CDP-Star™ was a chemiluminescent substrate either by using the GeneImages™ CDP-Star™ detection module following the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, UK) or by using the non-isotopic chemiluminescent enhanced probe system (NICETM) following the producer's instructions.

For visualisation the filters were placed against Hyperfilm (Amersham Pharmacia Biotech, Buckinghamshire, UK) at room temperature for 10–60 minutes. After probing the filters were stripped with 0.1% SDS and kept moist before hybridisation using the other probes. The same blots were hybridised with all probes used. Autoradiographs were scored over the well-resolved regions (1–23 kb) by eye by two independent assessors. The GelWorks1D Advanced - computer program (Phoretix International, Newcastle upon Tyne, UK) was used to estimate the sizes of the detected alleles on the basis of the sizes of the λ/*HindIII*-markers included in all gels. Mutants were identified as novel DNA fragments present in the children that could not be ascribed to either parent. The mutations were studied assuming that they were derived from the parental allele closer in size. Only mutations identified in two independent experiments (beginning from the DNA) were scored. The samples were decoded only after finishing all the analyses.

4.5 Statistical analyses

Conditional logistic regression methods were used in data analysis. The unit (stratum) used in the analysis was a family, i.e., a comparison of minisatellite mutation frequency was made between siblings born prior to and following cleanup work in Chernobyl. Hence, children born to the same mother and father were compared with each other. The outcome event was a minisatellite mutation, i.e., children with confirmed minisatellite mutations were regarded as cases and those without mutations as controls. Explanatory variables included exposure status (pre- vs. post-Chernobyl children), father's age and radiation dose, and the sex of the child. Statistical significance was evaluated using two-sided likelihood ratio tests and confidence intervals were likelihood-based. In addition, the doubling dose was calculated using unconditional logistic regression methods including only the post-Chernobyl children as subjects and continuous dose as the explanatory variable. The statistical analyses were carried out using the STATA statistical computer program package.

5 Results and discussion

5.1 The usability of PCR-based minisatellite mutation screening method

The usability of PCR-based minisatellite mutation screening methods in family studies was tested in 192 families of Estonian Chernobyl cleanup workers and in 48 Estonian control families. Altogether 886 individuals were studied using five minisatellite loci (*APOB, HRAS, MCOB19, MCT118*, and *YNZ-22*) in PCR-based analysis. The results obtained revealed children with mutations in several of the studied loci. When a child had a minisatellite mutation in three or more of the studied loci, paternity was considered excluded. Non-paternity was detected in 6.2% (12/192) of the exposed fathers and in 8.3% (4/48) of the non-exposed fathers from the additional control group.

In addition to minisatellite mutations connected with non-paternity, only one paternal mutation (*APOB*: father's allele + 2 repeats) was detected in the PCR-based minisatellite mutation screening. The father of this child belonged to the additional control group and was thus unexposed to radiation. No mutations (except those connected with non-paternity) were detected in the exposed families with the PCR-based method. As expected, the studied minisatellite loci proved unuseful in mutation screening due to their low spontaneous mutation rates, which however were the prerequisite for reliable paternity testing.

The use of the PCR-based method was very easy and quick with the short alleles but with the longer alleles, other PCR-conditions had to be developed to accomplish reliable amplification of the DNA samples. Despite using the PCR-conditions suitable for the longer alleles, the amplification of the few very longest alleles was difficult and unreliable thus causing many repetitions of the assay. However, the PCR-based method revealed non-paternity very efficiently and it can be used in testing the paternity of children before the actual mutation screening takes place, thus avoiding the laborious Southern blot-based analyses of families with non-paternity. Therefore, the PCR-based minisatellite mutation detection method was not shown to be recommendable for minisatellite mutation screening due to several existing long alleles at minisatellite loci most suitable for mutation screening (high spontaneous mutation rates), but it was applicable to the efficient ascertainment of the paternity of the children. This result is in concordance with the most popular use of PCR-based micro- or minisatellite loci in paternity or forensic testing, where the aim is to identify an individual, not a mutation.

5.2 Minisatellite mutation rates among children of Estonian Chernobyl cleanup workers

The parental origin and germline length change were determined for 94 *de novo* mutations (52 mutations among 155 exposed children and 42 mutations among 148 reference children) found at the eight tested minisatellite loci in the 147 studied families of the Estonian Chernobyl cleanup workers (Table 12). Only mutations of paternal origin were used as the endpoint in the publication (Kiuru et al. 2003). Of the eight single locus probes used, CEB1 had expectably the highest mutation rate. The CEB1 mutation rate (~14/100 paternal meioses) detected in post-Chernobyl children was comparable with the results found in other radiation exposed European populations [11/100 (Dubrova et al. 1997), 15/100 (Livshits et al. 1999, 2001), and 17/100 (Dubrova et al. 2002b)]. The gender of the child had no effect on the minisatellite mutation rates (OR 0.95, 95% CI 0.50-1.79). However, there was no reason to assume a shift in sex ratio because minisatellites are not X-linked lethal mutations shifting the sex ratio in favor of female offspring.

The detected minisatellite mutations were studied further by assuming that the progenitor allele was the paternal allele closest in size to the mutant allele (Jeffreys et al. 1988). As the same men fathered both groups of the studied

Table 12. Paternal minisatellite mutation frequency among pre- and post-Chernobyl children.

	Pre-Chernobyl children			Post-Chernobyl children		
Probe	**No. of mutations**	**No. of paternal meioses**	**Mutation frequency**	**No. of mutations**	**No. of paternal meioses**	**Mutation frequency**
B6.7	10	146	0.069	9	153	0.059
CEB1	15	148	0.101	21	155	0.136
CEB15	4	148	0.027	7	155	0.045
CEB25	4	148	0.027	4	155	0.026
CEB36	2	148	0.014	2	155	0.013
MS1	4	148	0.027	6	155	0.039
MS31	1	148	0.007	2	155	0.013
MS32	2	148	0.014	1	155	0.006
Total	42	1 182	**0.036**	52[1]	1 238	**0.042**

[1]In addition to these 52 mutations, 8 mutations (2 at B6.7, 4 at CEB1, 1 at CEB15 and 1 at MS1) were detected in post-Chernobyl children in 22 families, which were excluded from the publication due to the missing pre-Chernobyl children. The addition of these mutations to the other mutations of post-Chernobyl children (60/1414 = 0.042) would not have changed the detected mutation frequency of 0.042.

children, the sizes of progenitor alleles were identical in both groups, which allowed further comparison of mutation spectrum. The distribution of the detected gained and lost paternal minisatellite repeats at the studied eight minisatellite loci in the pre- and post-Chernobyl children is illustrated in Figure 4. Most of the detected minisatellite mutations both in the pre- and post-Chernobyl children were small changes in the length of the repeated minisatellite allele. A few larger changes in the number of repeats were seen both in pre- and post-Chernobyl children. The results obtained in the present study are in agreement with the results of previous studies analysing spontaneous mutations at these minisatellite loci (Jeffreys et al. 1988, 1984; Buard et al. 1998; Tamaki et al. 1999; Vergnaud and Denoeud 2000), most mutation events involved the gain or loss of only few repeat units. The distribution of length changes was similar between the two groups of children. Therefore, we conclude that there is no obvious difference in the spectrum of paternal minisatellite mutations between the pre- and post-Chernobyl children.

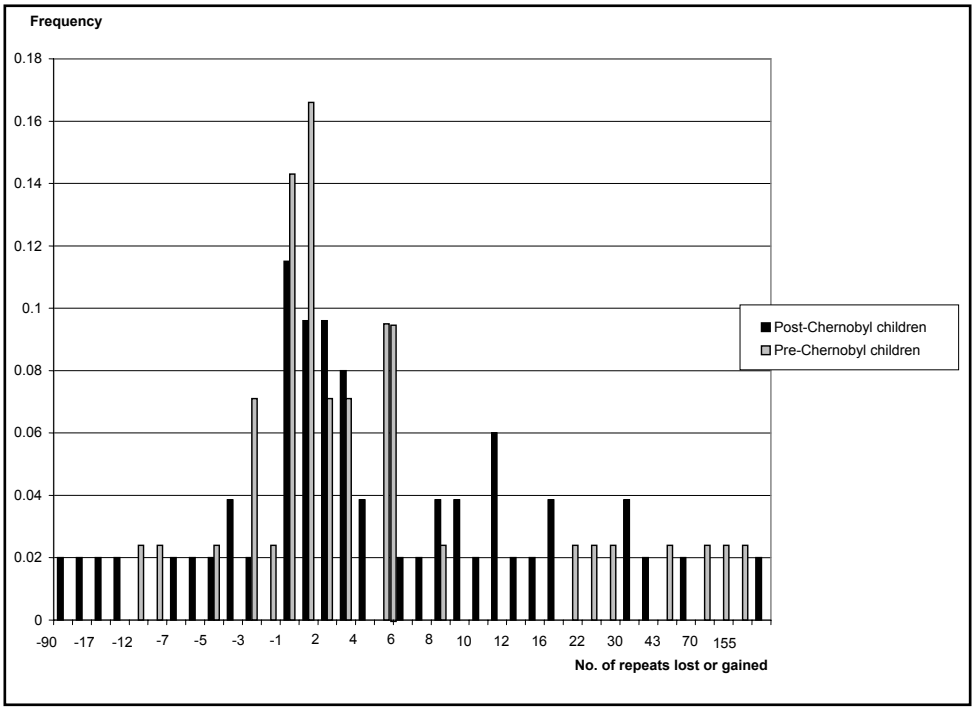

Figure 4. Detected lost and gained minisatellite repeats at the studied eight minisatellite loci.

5.3 Effect of the father's radiation exposure on minisatellite mutation rates

When the effect of the father's exposure to minisatellite mutation rates was examined, it was shown that the minisatellite mutation rate was higher in the post-Chernobyl than it was in pre-Chernobyl children in six out of eight used loci. The largest difference was a two-fold increase of minisatellite mutations at locus MS31 (Table 13). However, it may well be a chance finding, as it resulted from one and two mutations in the unexposed and exposed groups (Table 12). No single locus showed a statistically significant increase in the mutation rate among exposed children. There was a small, non-significant increase in the combined single locus probe mutation frequency among the post-Chernobyl children compared with the pre-Chernobyl children (0.042 versus 0.035, Table 13). In the logistic regression analyses, an odds ratio of 1.3 (95% CI 0.8-2.2) (and a rate ratio of 1.2) was obtained.

Table 13. Paternal minisatellite mutation frequency among pre- and post-Chernobyl children with odds ratio (and 95% confidence interval, CI).

	Mutation frequency		
Probe	**Pre-Chernobyl children**	**Post-Chernobyl children**	**Odds ratio[1] (95% CI)**
B6.7	0.069	0.059	1.00 (0.35-2.85)
CEB1	0.101	0.136	1.38 (0.68-2.80)
CEB15	0.027	0.045	1.65 (0.48-5.68)
CEB25	0.027	0.026	1.00 (0.25-4.00)
CEB36	0.014	0.013	0.84 (0.12-6.12)
MS1	0.027	0.039	1.41 (0.40-5.02)
MS31	0.007	0.013	2.00 (0.18-22.1)
MS32	0.014	0.006	0.50 (0.05-5.51)
Total	**0.036**	**0.042**	**1.33** (0.80-2.20)

[1]Odds ratios were obtained using conditional logistic regression, i.e., comparison between siblings within a family, therefore the odds ratios need not correspond to group frequencies.

The lack of a statistically significant increase in the minisatellite mutation frequencies could be due to too small sample size. However, if the total number of persons (N) needed for the detection of a 2-fold increase in minisatellite mutation rate is calculated by Eq. (3) using m_0 of 10^{-2} (from Table 3) (detected minisatellite mutation frequencies from Table 10 cannot be used here because they are maximum frequencies, not mean rates), then $N = 12/(8 \cdot 10^{-2}) = 12/0.08 = 150$. This result indicates that a 2-fold increase in the minisatellite mutation rate could have been detected by studying 150 case and 150 control children. Therefore, the 155 case and 148 control children studied in the present study should form an adequate sample for detecting a 2-fold increase in the minisatellite mutation rates. In addition, according to power calculations, the studied material and the minisatellite loci used in the present study were sufficient for detecting a 1.5-fold increase in the mutation rate in the eposed group with 95% probability. Therefore, it seems that the used material and minisatellite loci should have detected a 2-fold, even a 1.5-fold increase in minisatellite mutation frequencies. However, the fathers studied were exposed to so low doses of radiation that only a 1.3-fold increase in paternal minisatellite rates was detected. Such a small increase in the mutation frequency may be under the detection limit of the used study design and either a bigger sample or higher radiation exposure would have been needed in order to reach statistical significance.

In this study, the failure to detect a significant increase in the mutation frequency in men receiving 109 mSv of radiation is consistent with the absence of an increase in minisatellite mutations in the offspring of persons exposed to radiation from atomic bombs (Kodaira et al. 1995; Satoh and Kodaira 1996; Satoh et al. 1996) and in the Ukrainian population exposed by the Chernobyl disaster (Livshits et al. 1999, 2001) as well as with the failure to detect increases in mutations by SP-PCR in the sperm of men receiving radiotherapy (May et al. 2000), mutagenic therapy (Armour et al. 1999), or cancer chemotherapy (Zheng et al. 2000). However, an increase in minisatellite mutations has been found in the Belarusian (Dubrova et al. 1996, 1997) and Ukrainian (Dubrova et al. 2002b) populations exposed to the Chernobyl radioactive fallout as well as in the population living around the Semipalatinsk nuclear test site in Kazakhstan (Dubrova et al. 2002a), where 1.5-, 1.6-, and 1.8-fold increases in minisatellite mutation rates have been detected (respectively) (Table 5).

It is not possible at present to explain the differences between these human studies, but it should be noted that all data on minisatellite mutation induction in humans are derived from a relatively small number of people exposed to different sources of ionising radiation. This variety of sources currently prevents any reliable comparisons between most of the results of these studies. However, the source of ionising radiation is comparable in the present study and in the study

by Livshits et al. (2001) because the Chernobyl cleanup workers were exposed to repeated small daily doses of ionising radiation. The results of these studies are in concordance as Livshits et al. (2001) detected a 0.9-fold paternal minisatellite mutation rate and the corresponding value was 1.3 in the present study. Previous studies in male mice have clearly shown that the yield of germline mutations after a repeated external fractionated radiation exposure was less than when the same dose was given in a single exposure (Lyon et al. 1972). Given that he maximum reported dose to the Chernobyl cleanup workers of 250 mGy is below any known estimates of the doubling dose for mice (Dubrova et al. 1998a, b; Sankaranarayanan and Chakraborty 2000a) and assuming the dose-fracionation effects, the expected increase in mutation rate in this group may be too small to be realistically detected. Therefore, the results obtained in the present study seem to show that there is no big increase in the examined minisatellite mutation rates due to a father's radiation exposure, presuming that the increase in the minisatellite mutation rate in the studied Estonian cohort was not too tiny to detect. Alternatively, it is possible that the lack of minisatellite induction is a result of the duration of exposure. Since the majority of male germline cells are DNA repair-proficient (Adler 1996) the damage induced by discrete radiation exposure may be repaired preventing minisatellite hyper-instability.

The studies by Dubrova et al. (1996, 1997, 2002a, b) differed from the present study as their populations were exposed largely internally and chronically and the maternal as well as paternal germ cells, and the developing embryo and foetus, were all exposed to radiation. In the first two studies by Dubrova et al. (1996, 1997) multilocus probes (33.15 and 33.6) were also used which cannot distinquish paternal and maternal mutations from each other. In these studies, the control population was from the United Kingdom, which has a different genetic background than the exposed population. When only the paternal single locus minisatellite mutations were taken into consideration, the minisatellite mutation frequency increased 1.5-fold (Dubrova et al. 1997) (Table 5). The induction of minisatellite mutation in Belarus, Kazakhstan, and Ukraine (Dubrova et al. 1997, 2002a, b) may reflect inability to repair DNA damage arising from constant internal low level exposure.

In the Japanese atomic bomb survivor studies (Kodaira et al. 1995; Satoh and Kodaira 1996; Satoh et al. 1996), radiation exposure was acute gamma and neutron irradiation and conception occurred long after the exposure. Therefore, the mutation rate may have decreased back to the normal level with the passage of time. This was shown to occur in the 3-generation study of the Kazakhstan population around the Semipalatinsk nuclear test site, where the increase in the minisatelllite mutation rate was 1.8-fold in the highly exposed P_0-generation and it decreased to 1.5-fold level in the much less ex-posed F_1-generation (Dubrova

et al. 2002a) (Table 5). In the present study, all children were conceived quite close to the radiation exposure of the father. Even the youngest children were conceived within three years of the father's exposure. Thus minisatellite mutation frequencies have not had time long enough to return to normal levels and despite this, the mutation frequencies detected in the present study were not higher than 1.2-fold. However, Livshits et al. (2001) detected that the CEB1 minisatellite mutation frequency among Ukrainian Chernobyl cleanup workers fell from 18% to 13% among children conceived up to 2 months or at least 4 months (respectively) after the father had stopped cleanup work at Chernobyl. In the present study, 21 children were conceived up to 2 months and 133 children were conceived at least 4 months after the father has returned from cleanup work at Chernobyl. CEB1 mutations were detected in these groups in 5% and 13% of the children. Therefore, in the present material, the fast decrease of minisatellite CEB1 mutations could not be detected, but the result may be biased due to the small number of children conceived before 2 months after father's return from Chernobyl.

In the Ukrainian study (Dubrova et al. 2002b), 16% (27/171) of the studied families included children born before and after the Chernobyl accident. Results from these 27 families were not given separately in the publication, but according to Dubrova paternal minisatellite mutation rate was 0.0415 in the control group and 0.0585 in the exposed group among the 27 families. These mutation rates give a mutation rate ratio of 1.4 which does not differ substantially from the mutation rate ratio of 1.6 detected in the whole material. However, the present study is so far the only one in which a perfect match of exposed and unexposed children has been possible in each of the studied families. This was achieved by using intrafamily controls, i.e., comparing the children born after Chernobyl to children born before Chernobyl in the same family. This study design guaranteed that the genetic and environmental factors in the studied children were as identical as possible and that the individual minisatellite mutation rates (depending, e.g., on the repeat length) varying from father to father were the same between the children compared.

Because of the ideal study design of the present study, it was possible to compare children from the same families and it was detected that 16% (7/44) of the fathers having a minisatellite mutation in the post-Chernobyl child had it also in the pre-Chernobyl child and 20% (7/35) of the fathers having a minisatellite mutation in the pre-Chernobyl child had it also in the post-Chernobyl child. In 5 of these 7 sibling pairs, the mutations were at different minisatellites in pre-and post-Chernobyl children and in 2 sibling pairs, mutations were at the minisatellite CEB1 in both children. In addition, 9 post-Chernobyl and 7 pre-Chernobyl children had two different minisatellite mutations. These figures

may reflect the detected phenomenon of individual sensitivity to the induction of minisatellite mutations (inter-individual variation in mutation frequency) (May et al. 2000). The similarity of these figures in pre- and post-Chernobyl children also casts some doubt over the causality of the low radiation exposure of the father to these detected minisatellite mutations.

5.4 Effect of the father's radiation dose on the minisatellite mutation rates

For testing the correlation of the minisatellite mutation rates and the father's radiation dose, possible radiation-induced minisatellite mutations were explored by subdividing the cleanup workers according to their radiation dose. Children born to fathers with estimated doses of 200 mSv or more had a higher, but statistically non-significant increase in the minisatellite mutation rates (OR 3.00, 95% CI 0.97-9.30) compared with those with lower recorded doses (Table 14). Even though the result did not quite reach statistical significance, this was the strongest evidence we found for the hereditary effects of radiation.

Even the highest estimated doses (300 mSv) were moderate and for the examination of the question if dose rate could be critical; the minisatellite mutations were further explored by subdividing the cleanup workers according to their radiation dose rates (Table 15). By doing this, the results did not change substantially. Children born to fathers with the highest estimated dose rates of 5-6.8 mSv/d had a higher increase in the minisatellite mutation rate (OR 3.50) but showed a poorer significance (95% CI 0.73-16.80) than the group with the highest estimated dose (95% CI 0.97-9.30).

Table 14. Number of minisatellite mutations and odds ratios in relation to a father's estimated dose.

Father's estimated dose	Pre-Chernobyl children[1]	Post-Chernobyl children[1]	Odds ratio (95% CI)
43-99 mSv	16/61	17/66	0.95 (0.44-2.05)
100-199 mSv	12/48	14/52	1.14 (0.47-2.77)
200-300 mSv	6/37	13/35	**3.00** (0.97-9.30)

[1]Number of children with minisatellite mutation(s)/total number of children.

Table 15. Number of minisatellite mutations and odds ratios in relation to a father's estimated dose rate.

Father's estimated dose rate	Pre-Chernobyl children[1]	Post-Chernobyl children[1]	Odds ratio (95% CI)
0.004-1.9 mSv/d	25/101	29/110	1.08 (0.59-1.97)
2.0-4.9 mSv/d	5/23	7/22	1.50 (0.42-5.31)
5.0-6.8 mSv/d	4/22	8/21	**3.50** (0.73-16.80)

[1]Number of children with minisatellite mutation(s)/total number of children.

Table 16. Minisatellite mutation risk in relation to a father's estimated dose in post-Chernobyl children (exposed group) alone.

Father's estimated dose	Odds ratio (95% CI)
43-99 mvS	1.00 (0.97-1.08)
100-199 mSv	1.04 (0.48-2.25)
200-300 mSv	**1.51** (0.70-3.44)

When the intrafamily control children (pre-Chernobyl children) were excluded and only the post-Chernobyl children ("exposed" group) were statistically tested, there was no significant correlation between the father's estimated dose and the frequency of minisatellite mutations (Table 16). There was some indication of a higher risk (OR 1.51) in the highest dose group, but it was statistically insignificant (95% CI 0.70-3.44).

Concerning dose response analysis, earlier studies on the induction of minisatellite mutations in mice have failed to produce any reliable relationships between radiation dose and mutation frequency (Dubrova et al. 1993; Sadamoto et al. 1994; Fan et al. 1995). However, later Dubrova et al. (1998a, b) showed a linear dose-effect relationship for mutations in pre-meiotic spermatogonia and stem cells, with a doubling dose of 0.33 Gy. The estimated doses and dose rates of the fathers in the present study were quite low. Only 25% of the fathers received a radiation dose of 200 mSv or higher and just 14% of the fathers received a dose

rate of 5.0 mSv/d or higher. The estimated doses were not absolute, but merely the best estimates of doses that could be obtained as all of the fathers did not wear a personal dosimeter while working at Chernobyl. As 75% of the studied fathers received low or moderate doses (<200 mSv), their doses were clearly below the doubling dose detected in mice and even further apart from the doubling dose of 1 Gy recommended for humans by UNSCEAR (2001). Thus, the lack of the correlation of the minisatellite mutation rates and the father's radiation dose in the present study may be due to low radiation exposure of the fathers as the doses were probably under the detection limit of the method used. However, the obtained results could be interpreted so that total radiation doses received were more critical for minisatellite mutation induction in the male spermatogenesis than the dose rate.

5.5 Effect of the father's age on the minisatellite mutation rates

Examination of the paternal age effect on the minisatellite mutation rates showed that the mutation rates were not associated with the father's age (OR 1.04, 95% CI 0.94-1.15). Human mutations are known to increase with increasing paternal age, but since we compared children of the same father (difference in father's age between children was 1.0–12.9 years, median 3.8 years), the effect of father's age was nearly the same in both (or all) children. Thus, due to colinearity with division into pre- and post-Chernobyl children, no meaningful adjustment for the effect of father's age was possible.

For further analysis of the paternal age effect, the children were individually divided into different subgroups according to their birth order (pre-Chernobyl children, post-Chernobyl children and additional control children) and mutation status (mutation, no mutation). There were no significant differences in the median ages of father's in these different subgroups (Table 17). In each subgroup of children, the median ages of the fathers were higher in the group of children without minisatellite mutation than in the group of children with minisatellite mutation. According to this study, there seems to be no father's age effect in minisatellite mutation frequencies. Age-related increase in paternal germline mutation rate has not been shown in human minisatellites (Livshits et al. 2001; Dubrova et al. 2002b), although such a trend has been detected at human protein-coding genes (Vogel and Rathenberg 1975).

Table 17. Median age of the father in different subclasses of children.

	Father's median age (y)	
	Children with minisatellite mutation	**Children without minisatellite mutation**
Pre-Chernobyl children	24.5	25.3
Post-Chernobyl children	29.3	30.3
Additional control children	27.0	28.1

5.6 Effect of the stage of spermatogenesis on the minisatellite mutation rates

When the effect of the stage of spermatogenesis on the minisatellite mutation rates was examined, no obvious differences were found in the time interval between the birth of the child and father's return from Chernobyl (Table 18). However, children born more than 49 weeks after the return date had the highest odds ratio (1.45, 95% CI 0.84-2.52). There were three children born 49–52 weeks (corresponding to exposure of spermatogonia) following the return date, all having a minisatellite mutation, while no mutations were found in their unexposed siblings. Due to the small number (n = 3) of these children, the result may well be a chance finding.

Human spermatogenesis lasts for 74 days (Figure 2) and sensitivity to mutagens varies during this time. Spontaneous minisatellite mutations occur at meiosis and it is therefore likely to be the most vulnerable stage of spermatogenesis. Also minisatellite mutation data show that minisatellite mutations in the paternal germline most likely occur at meiosis (Jeffreys et al. 1994; May et al. 1996; Jeffreys and Neumann 1997; Tamaki et al. 1999; Buard et al. 2000; Stead and Jeffreys 2000). If so, then exposure to ionising radiation could potentially affect the stability of minisatellite loci over a very short interval of meiosis. As protracted exposure would result in exposure at meiosis to successive gamete generations, it may induce mutations more effectively than acute exposure. To judge from the results of the present study, the meiosis appears to be highly unlikely candidate for the most vulnerable stage of the spermatogenesis. Given a relatively high initial exposure from the short-lived radionuclides, the preferential targeting of meiosis should result in

Table 18. Number of minisatellite mutations and odds ratios in relation to the time between father's return from Chernobyl and the date of a child's birth.

Time between father's return from Chernobyl and date of child's birth	Pre-Chernobyl children[1]	Post-Chernobyl children[1]	Odds ratio (95% CI)
38-44 weeks[2]	3/9	1/9	0.33 (0.03-3.20)
45-48 weeks[3]	3/11	3/11	1.00 (0.14-7.10)
>49 weeks[4]	28/126	40/133	**1.45** (0.84-2.52)

[1]Number of children with minisatellite mutation(s)/total number of children. [2]Stage of spermatogenesis assumed to be post-meiotic (spermatozoa, spermatids) when the gestation age at birth of the child was presumed to be 40 weeks. [3]Stage of spermatogenesis assumed to be meiotic (spermatocytes) when the gestation age at birth of the child was presumed to be 40 weeks. [4]Stage of spermatogenesis assumed to be pre-meiotic (spermatogonia, stem cells) when the gestation age at birth of the child was presumed to be 40 weeks.

a considerably elevated paternal mutation rate during the first year after the Chernobyl accident.

However, we found no substantial differences between children born in the first year or later following father's return from Chernobyl. If anything, the minisatellite mutation frequency tended to show a slight increase with time since exposure (at least until 52 weeks). The highest risk (OR 1.45, 95% CI 0.84-2.52) was observed in the >49 week category (Table 18) corresponding to exposure of spermatogonia and stem cells. It therefore appears that pre-meiotic diploid stem cells and spermatogonia could accumulate radiation-induced damage, elsewhere in genome, that subsequently affects the stability of minisatellite loci at meiosis. This would fit well with the concept (see 2.8.) that spermatogonial stem cells and resting oocytes have the highest risk of accumulating radiation-induced genetic damage (Searle 1987). This delayed stimulation of minisatellite mutation in meiotic cells is reminiscent of the phenomenon of radiation-induced genomic instability (see 2.6.3). However, it has to be kept in mind that the other categoria than the >49 week category were also far too small to reach statistical significance.

However, stages of spermatogenesis were not determined with optimal accuracy in the present study since it was not possible to obtain the actual pregnancy weeks at delivery and thus, the pregnancies were assumed to be full term pregnancies at 40 weeks. Pregnancies are not allowed to last over 42 weeks

(= 40 weeks from fertilisation) during maternity care, but pre-term pregnancies can naturally have deviation from the assumed 40 week duration of pregnancy. In boundary value cases, even a one-week change in the length of pregnancy can thus change the category of the stage of spermatogenesis to which an individual belongs to in Table 18.

5.7 The applicability of the linear dose response

The results of the present study could neither favour nor oppose the linear dose response model, as the statistical power of the present study was insufficient. Furthermore, the fact that changes in minisatellite repeat sequences can arise as a result of radiation damage outside the sequence concerned and the likely involvement of some sort of signal transduction in the minisatellite mutation process makes the determination of the nature of the radiation dose response impossible. However, Dubrova et al. (2002a) have detected a negative correlation of minisatellite mutation rate with the parental year of birth and thus the time passing from the exposure. They state that the correlation implies the presence of a dose response for minisatellite mutation induction and suggests that an elevated mutation rate in the affected families is indeed radiation-induced.

5.8 The doubling dose estimate

The individual dosimetry used in this study potentiated the assessment of the doubling dose. In linear unconditional analysis, the doubling dose of minisatellite mutations was estimated at 0.5 Sv, but there was considerably uncertainty (95% CI 0.1 mSv-∞). The statistical power of this study was insufficient to give a reliable estimate of the doubling dose due to the relatively small number of mutations observed and the limited range of exposure. Therefore, the present minisatellite study could not give more precise estimate of the assumed germline doubling dose of round 1 Gy for germline mutation in humans (Sankaranarayanan and Chakraborty 2000a; UNSCEAR 2001).

5.9 Comparison of paternal and maternal minisatellite mutation rates

In addition to paternal mutations, maternal mutations were also detected in the studied Estonian families, where the father of the family had been exposed to radiation during the cleanup work at Chernobyl (Table 19). The *B6.7* and *CEB1* mutations frequently seen in males were rarer in females where most mutations were detected, as expected (Table 10), at the *MS1* minisatellite locus.

Table 19. Maternal minisatellite mutation frequencies among children in paternally exposed Estonian families

	Pre-Chernobyl children			Post-Chernobyl children		
Probe	**No. of mutations**	**No. of maternal meioses**	**Mutation frequency**	**No. of mutations**	**No. of maternal meioses**	**Mutation frequency**
B6.7	2	146	0.014	3	153	0.020
CEB1	1	148	0.007	0	155	0
CEB15	1	148	0.007	2	155	0.013
CEB25	2	148	0.014	3	155	0.019
CEB36	2	148	0.014	3	155	0.019
MS1	7	148	0.047	5	155	0.032
MS31	1	148	0.007	0	155	0
MS32	1	148	0.007	5	155	0.032
Total	17	1182	**0.014**	21[1]	1238	**0.017**

[1]In addition to these 21 mutations, there were 6 mutations (2 at CEB25, 2 at CEB36, 1 at MS1 and 1 at MS31) in post-Chernobyl children in 22 families, which were excluded from the published study due to the missing pre-Chernobyl children. The addition of these mutations would have caused only a minor increase (27/1414 = 0.019) in the detected mutation frequency of 0.017.

Maternal minisatellite mutations were more common than expected at locus *MS32* and less common than expected at locus *B6.7*. However, allele specific mutation frequencies and the small number of the offspring studied inabled closer comparisons of the minisatellite mutation rates.

In the present study, the maternal mutation frequency was 0.014 in the pre-Chernobyl children and 0.017 in the post-Chernobyl children. Therefore, the maternal mutation frequencies found were less than half the frequencies detected in the father's germline (Table 12). This is in concordance with the observations in the literature that minisatellite mutations are less frequent in females (Table 10). However, both the maternal and paternal minisatellite mutations were increased 1.2-fold in the post-Chernobyl children compared to their pre-Chernobyl siblings in spite of the fact that the fathers, not the mothers, were exposed to radiation. This could be explained by the low and fractionated radiation doses of the fathers making it impossible to detect any effects of radiation in minisatellite mutation rates. Therefore, mothers as well as fathers could be considered as non-exposed.

Maternal minisatellite mutations have not been as extensively studied as the paternal ones, but published maternal minisatellite mutation rates are in concordance with the results of the present study. Dubrova et al. (1997) detected a doubling of the maternal minisatellite mutation rate in Belarus from 0.009 in the controls to 0.018 in the exposed group. However, their controls cannot be regarded ideal because they were from the United Kingdom. Therefore, Dubrova prefers comparison between the exposed group in Belarus and a control group in Ukraine. When this is done with the maternal mutation rates of 0.0188 and 0.0143 then a 1.32-fold increase is detected in mutation rate in this group. However, the confidence interval of this ratio is wide (95% CI 0.43-2.69), basically meaning that there is not enough statistical power to test any hypothesis. In another study by Dubrova et al. (2002b), mutation rate in the germline of exposed mothers from Ukraine was not elevated, i.e., it was 0.0143 in the control group and 0.0145 in the exposed group. However, given again the wide confidence interval of the ratio of maternal mutation rates in the exposed group to control group in that study (95% CI 0.38-2.00), the authors could not exclude the possibility of an elevated mutation rate among the irradiated mothers.

Currently, there are no studies specifically examining the effect of maternal exposure to radiation on minisatellite mutation rates. The main problem with maternal minisatellite mutation studies is the fact that minisatellite mutation rates in the maternal germline are quite low (Table 10). Proper analysis of maternal minisatellite mutation rates would require a study containing at least 1 000 offspring from each group. Therefore, the present study as well as the former studies (Table 5), were all too small to provide enough statistical power for detecting an elevated maternal mutation rate in the exposed cohorts. It remains to be seen whether pre-meiotic, meiotic, and post-meiotic female germ cells respond in a manner similar to the male germline for minisatellites and whether similar mechanisms operate in male and female germ cells.

5.10 Minisatellite mutation rates in other Estonian control families

In the additional 44 control families from Estonia, 13 paternal mutations were detected at the eight tested minisatellite loci, giving a mutation frequency of 0.037 (Table 20). These additional control families gave an external reference rate for assessment of generalizability (selection). The observed mutation frequency was in concordance with the mutation frequency of 0.036 detected in pre-Chernobyl children (Table 13). Thus, these results affirmed that the study group used reflects the general spontaneous minisatellite mutation frequency in Estonia. Interestingly, no mutations were observed at minisatellite locus *B6.7* in these

Table 20. Paternal and maternal minisatellite mutation frequencies among children in the additional control families.

	Paternal minisatellite mutations			Maternal minisatellite mutations		
Probe	**No. of mutations**	**No. of paternal meioses**	**Mutation frequency**	**No. of mutations**	**No. of maternal meioses**	**Mutation frequency**
B6.7	0	44	0	0	44	0
CEB1	8	44	0.182	0	44	0
CEB15	1	44	0.023	1	44	0.023
CEB25	1	44	0.023	1	44	0.023
CEB36	0	44	0	1	44	0.023
MS1	2	44	0.045	3	44	0.068
MS31	1	44	0.023	1	44	0.023
MS32	0	44	0	1	44	0.023
Total	13	352	**0.037**	8	352	**0.023**

additional control families compared with 6.9% and 5.9% mutation frequencies among pre- and post-Chernobyl children (Table 12). On the other hand, the paternal CEB1 mutation frequency was higher among additional control children (18%) than among the pre-Chernobyl control children (10%). Furthermore, 8 maternal mutations were found in these additional control families, giving a mutation frequency of 0.023 (Table 20), which is somewhat higher than the observed maternal mutation frequency (0.017) in the exposed families.

6 Conclusions and future prospects

Minisatellites provide the most powerful tool currently available for monitoring heritable DNA mutations caused by ionising radiation or genotoxic agents. In the present study, a minisatellite mutation screening system using eight single locus probes was evaluated for the assessment of radiation-induced hereditary risks in humans. The PCR-based mutation screening method turned out to be a fast and reliable method for paternity testing. The minisatellite loci used efficiently revealed non-paternity. However, the PCR-based minisatellite method was not applicable to the mutation screening of loci with the highest spontaneous mutation frequencies, since an unacceptable amount of alleles were too long to be reliably amplified with PCR.

Therefore, the rates of heritable minisatellite mutations in the radiation-exposed population of Estonian Chernobyl cleanup workers were examined with the Southern blot-based minisatellite mutation screening system. It was shown that the minisatellite loci used in this study were well suited for mutation detection and the rate of minisatellite mutations was easily and reliably measured by the used eight single locus minisatellite probes, as all the detected mutations were confirmed in two independent experiments. The high frequency of spontaneous and induced mutations at these minisatellite loci allowed mutation induction to be evaluated at low doses of exposure. Dubrova states that the increase in minisatellite mutation rates that he and his collegues have detected in several populations is indeed caused by radiation exposure of the studied persons. It seems possible that minisatellite mutations are good indicators of radiation exposure. The nature of the damage signal created by radiation, whether in DNA or some other sensor molecule, and the mechanism of subsequent induced mutagenesis, remain major goals of future research. As a few minisatellites are also situated near important disease-related genes (see 2.2.1.5), the induction of minisatellite mutations should not be ignored in the context of potential hereditary risks.

The minisatellite mutation risk of children born after Chernobyl showed to be somewhat higher than the mutation risk of children born before Chernobyl, but statistically significant differences were not shown. The total radiation doses received by the fathers appeared to be somewhat more important for the minisatellite mutation induction than the dose rates they received, but no significant correlation between the father's estimated dose and the frequency of minisatellite mutations was detected. Testing of the applicability of the linear dose response model to this material was not possible. In addition, the doubling dose based on the material used in the present study contained considerably uncertainty.

However, statistically significant differences in the amount of minisatellite mutation induction between exposed and unexposed fathers could not be reached with doses below 200 mSv. The studied minisatellite mutation screening system enabled germline mutation detection in humans and more significant results would perhaps have been obtained if the radiation exposure had been higher. In other Estonian control families, the minisatellite mutation rates were shown to correspond to mutation rates in pre-Chernobyl control children as expected because they were non-exposed to radiation. The maternal mutation rate was of the same magnitude in all studies of Estonian families.

As expected, the paternal minisatellite mutation rates were shown to be higher than the maternal ones. However, the paternal age effect could not be seen in the detected minisatellite mutation frequency and due to the lack of knowledge of the absolute length of the pregnancies, no definitive conclusions could be drawn considering the effect of the stage of the spermatogenesis in the minisatellite mutation rate of the radiation-exposed men.

In summary, minisatellite mutation rates were compared within a family between the children born prior to and after the father was exposed to radiation while working in the cleanup activities at Chernobyl. Thus, the optimal reference material for genetic and environmental factors was used. The minisatellite screening system used had several potential advantages for monitoring germline mutation in men compared with standard genetic approaches. The probable selective neutrality of the minisatellite mutants used ensured that the mutants were not lost prenatally, as can occur at protein coding loci. The very high spontaneous mutation rates made it, in principle, possible to detect induced mutations in a relatively small population sample.

The application of minisatellite loci to the radiation risk assessment still requires much research and validation. Further application of minisatellites as a reliable indicator of the genetic consequences of radiation exposure is currently limited mainly because the mechanisms for the induction of minisatellite mutation by ionising radiation remain unknown. Minisatellite mutations do not confer hereditary defects, i.e., they themselves are not adverse genetic effects. As far as it is known, minisatellite mutations cause no known decline in the fitness or health of their carriers. An understanding of the mechanisms involved in induced minisatellite instability is required before any attempts to postulate on hereditary risks can be made. Additional studies are needed in order to clarify whether an increase in minisatellite mutations will or will not be indicative of an increase in mutations at some other, more essential genomic regions that do lead to human diseases. Given the unusual mechanism of germline instability resulting from gene conversion or recombination, and the indirect nature of induced mutations, it is not intuitive how induced instability may relate

to instability at other genome regions. It is possible that the indirect nature involves alterations in gene expression or repair enzymes, which may similarly affect other areas of the human genome. However, showing this would require germline mutagenicity studies, which in turn require a precise understanding of the timing of mutation induction and an evaluation of dose response parameters. These data are difficult to obtain due to current lack of appropriate animal models for studies on minisatellites. Also, the different mutation mechanisms that act in somatic cells prevent the extrapolation of results from human somatic cell cultures to germ cells. Unfortunately, the current study on minisatellite mutations in Estonian post-Chernobyl families clarified neither the timing of mutation induction nor the dose response parameters.

Among the 30 000 genes in the human genome, some genes can possibly respond directly to radiation exposure, but they have not yet been found. Since radiation mostly produces multigene deletions, it would seem plausible that those human processes that depend on the greatest number of genes would be the ones most vulnerable to the loss-of-function of several genes. Embryonic development and brain function are two human processes meeting these criteria. Potential phenotypes could share some common features such as multisystem developmental abnormalities or mental retardation. However, these phenotypes are not easily differentiated from corresponding phenotypes caused by some other reason. As the minisatellites are so far the only model for efficiently studying human heritable mutations directly, it would be sensible to explore the induction of minisatellite mutations after irradiation and the possible consequences of these mutations for human health. The development of human molecular genetics will give better understanding of changes and their function in so far poorly known phenomena, e.g., individual radiosensitivity, genomic instability, and the bystander effect, thus potentiating the more reliable assessment of radiation risks.

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