New methods for assessing male germline mutations in humans and genetic risks in their offspring

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Keywords: germline mutagenesis, paternal exposure, ESTRs, minisatellites, RAPD-PCR
Running title: Methods for assessing male germline mutations in humans

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Abstract

Germline mutations resulting from chemical or radiation exposure are a particular problem in toxicology as they affect not only the exposed generation, but an infinite number of generations thereafter. Established methods to show that these mutations occur in an F1 or subsequent population require the use of a large number of progeny for statistical significance. Consequently, many thousands of animals have been used in the past. Such a use is no longer considered desirable and is also very expensive. Several new molecular techniques (including analysis of tandem repeats and randomly amplified polymorphic DNA) now provide alternative methods of assessment, which also allow the quantification of individual mutations in individual sperm cells. These can also be applied to human offspring, making extrapolation obsolete. The downside of these methods is that they effectively determine the mutation rate in certain regions of DNA and the relevance of these to diseases, particularly cancer, is not always apparent. Therefore it must be assumed that an increase in mutation rates in these selected regions correlates with altered phenotype. However, disease types linked to changes in tandem repeat length indicate that these may act as relevant markers for the development of phenotypes. Further research and evaluation is required to more closely link changes in DNA with altered phenotype and validate the use of tandem repeats and randomly amplified polymorphic DNA in transgenerational genotoxicity testing. This paper introduces and compares recently developed methods to assess mutations in sperm due to stem cell damage.
Introduction

New methods are needed to detect germline mutations induced by exposures to genotoxins in humans. Too little is known about how genotoxic compounds induce mutations in human germ cells and the subsequent transmission of these to offspring. Transmittable genetic damage in humans has been studied in populations exposed to radiation (1), whereas germline mutations induced by environmental pollutants have only been studied in animals. Most of the mutations transmitted to offspring had a paternal origin (2-5). A similar result might be expected in humans although a direct relationship between mutations in human sperm and a genetic disease induced in the progeny has not been reported yet (6).

A parallelogram approach developed by Sobels (7) and extended by Adler (8) has been used until now to extrapolate measurable effects in rodent germ cells, their progeny and human germ cells in vitro to estimate the genetic risks in humans in vivo (Figure 1). This approach uses classical methods, such as the morphological specific locus (MSL) test and the dominant lethal test. These tests have low sensitivity, use large numbers of progeny to obtain the recommended sample size, and are very time consuming and expensive. Recently developed germ cell mutagenicity tests seem to have rendered this parallelogram approach obsolete, since these new tests can detect mutations in human germ cells and offspring, and are suitable for studying genetic risks in human populations. A recent review by Singer et al. (9) compared classical approaches (e.g. MSL and heritable translocation assay) with new methodologies (e.g. expanded simple tandem repeats (ESTRs) mutation assay and the gene mutation test in transgenic rodents) but too little data was available to compare the performances of these tests in detail.

Of these new techniques, only the analysis of tandem repeat sequences seems suitable for testing in humans. We will therefore discuss this assay in greater detail together with randomly amplified polymorphic DNA PCR (RAPD-PCR), an assay that could be used to quickly screen exposed humans for the transmission of mutations to their offspring. Although these new methods seem to have a high sensitivity, they have yet to be validated and only a few laboratories are equipped to perform the analysis of tandem repeat sequences.
Germ cell tests play a decisive role in classifying and labelling chemicals for genotoxicity. To minimize animal use, tests that detect effects in germ cells should be performed first. However, if quantification of these effects is required, assays for mutations in the offspring are essential. In the guidance document for genotoxicity under Registration Evaluation and Authorisation of Chemical Substances, the new European Community (EC) policy for authorization of new and existing chemicals, a test that determines changes in hypervariable tandem repetitive regions is stated as an alternative to classical tests. As this test also allows the measurement of heritable damage in humans, it is a recommended alternative.

Germline mutagenicity tests

Randomly amplified polymorphic DNA PCR

RAPD-PCR was first described by Williams et al. and is based on the amplification of random unknown DNA segments with a single primer of arbitrary nucleotide sequence (10). RAPD-PCR can be used to detect genomic alterations without prior information about the loci. Multiple fragments are generated in a single reaction that can be visualised after gel electrophoresis (Figure 2A). Any genomic alteration (point mutations, structural rearrangements, deletions, insertions, etc.) may change the place where the primer anneals, resulting in different profiles (Figure 2B and C). For germ cell mutagenicity testing, the patterns obtained from the mother and father can be compared with those from the offspring. Differences between the patterns may indicate a genomic alteration if 1) the DNA fragment is only visible in the pattern of the child, 2) the DNA fragment is present in the pattern of the father and/or the mother, but absent in the pattern of the child, or 3) bands are increased or decreased in intensity. However, PCR products that disappear or change in intensity should be interpreted with care, because of the possibility of heterozygosity in the parents. Other PCR-based techniques have also been developed, like DNA amplification fingerprinting (DAF) and arbitrarily primed PCR (AP-PCR) (11,12). However, these have yet to be used for germline mutation detection.

RAPD-PCR might detect single base changes in genomic DNA (10) although genomic rearrangements are probably detected the most (13). As RAPD-PCR only detects genomic alterations in or close to a priming site, the use of multiple primers may
increase the sensitivity of this method. Moreover, the sensitivity of the test could be improved by using a more accurate method described by Valentini et al. for the separation and detection of multiple amplified fragments (14). In toxicological exposure experiments to assess population-genetic effects this method successfully detected genomic alterations induced by benzo(a)pyrene (B[a]P), copper, mitomycin C, UV radiation and X-rays (15-18). Theoretically, all routes of exposure in which toxicants may affect the genetic structure can be analysed. Experimental studies usually require several hundred offspring for the powerful detection of germline alterations using approximately 5 to 10 RAPD primers.

RAPD-PCR has several advantages: 1) it is relatively inexpensive and rapid, 2) it yields information on a large number of loci, 3) the same primers can be used for genomic analysis in all species, including humans, and 4) it can be performed with relatively small sample sizes. However, there are also several drawbacks; 1) the results are considered to be qualitative and indicative, and should be further corroborated by cloning, sequencing and probing techniques, 2) the nature of the genomic change observed cannot be assessed from the sequence of the RAPD-PCR product, 3) the technique may not screen the genome as randomly as expected, and 4) both nuclear and mitochondrial DNA may be amplified during PCR. Amplification of mitochondrial DNA may complicate the interpretation of RAPD fingerprints as it is not a Mendelian marker, but only inheritable from the mother (19). Reproducibility of RAPD profiles is another potential pitfall (20). However, this can be overcome by using appropriate PCR conditions and by cleaving DNA with restriction enzymes prior to the amplification (21).

So far, RAPD-PCR has mainly been used in ecotoxicological studies, for example, to detect genomic alterations in Daphnia magna exposed to B[a]P (13,22). Weinberg et al. (23) have used the method in humans and they detected a seven-fold increase in mutation frequencies in blood from children of families conceived after parental exposure to radiation from the Chernobyl accident, compared to children conceived before parental exposure. They concluded that exposure to low dose radiation induces genetic changes in the germline. Although the finding of a seven-fold increase in mutation rate has been questioned by Jeffreys and Dubrova (24) and mutants have not
been validated, the study nevertheless raises major concerns regarding radiation-induced heritable genetic effects.

In conclusion, RAPD-PCR seems to be a promising method for the analysis of germline mutations in offspring. For human studies, however, PCR conditions and RAPD-PCR methods should be optimised to yield reproducible DNA fingerprints, and several verification methods should be used to validate and confirm results (13). Although the findings obtained by RAPD-PCR can only be regarded as indicative at present, the method can still be used as an effective screening tool in studying germline mutagenesis.

**DNA fingerprinting of tandem repetitive DNA sequences**

Part of the genome consists of tandem repetitive sequences, which are known to be unstable and predominantly non-coding DNA. These sequences can be divided into microsatellites, minisatellites and ESTRs based on sequence size and number of repeats (Table I). Yauk (25) provides further information on the differences between minisatellites and ESTRs with respect to their structure and mutational mechanisms.

Minisatellites and ESTRs can be sensitive tools for monitoring germline mutations after mutagenic exposure, since both exhibit high mutation rates. This facilitates the assessment of induced mutations in a relatively small number of samples following environmentally relevant exposure (26). The DNA is digested with restriction enzymes and subsequently electrophoresed, before being hybridised with probes that are complementary to hypervariable loci. This gives a unique pattern of fragments (DNA fingerprint) (Figure 3). The use of multiple probes can increase the probability of detecting mutations, when comparing the patterns between parents and their offspring (each band should also be present in one of the parents). Therefore this is a statistically powerful technique that reduces the time needed and costs involved to quantify the number of germline mutations.

Recent experiments have shown that minisatellites and ESTRs are able to detect an increase in germline mutations in humans and animals exposed to radioactive or chemical pollutants in their natural environment (2-5). Multi-locus DNA fingerprinting has also demonstrated significantly elevated mutation rates in the offspring of humans.
inhabiting the radioactive polluted areas of Belarus near Chernobyl, when compared to control families from the United Kingdom. Moreover, a significant radiation-dose effect relationship was observed (1,27).

These studies demonstrate that the analysis of tandem repetitive sequences could be a sensitive method for the detection of germline mutations transmitted to the next generation and caused by long term and/or low dose environmental exposure. However, very little is currently known about germline mutations induced at hypervariable tandem repeat sequences by specific chemicals. Two mono-functional alkylating agents, ethylnitrosourea (ENU) and isopropyl methanesulphonate (iPMS), and the anticancer drug etoposide have already been investigated and led to elevated ESTR mutation rates in the germline of male mice (28). Although these results demonstrate that tandem repeat sequences can be used to monitor germline mutation induction, the effects of exposure to environmental or dietary mutagens other than radiation on germline mutation induction at tandem repeat loci in rodents and humans clearly merits further evaluation.

Small-pool PCR
Small-pool PCR (SP-PCR), which is derived from DNA fingerprinting of minisatellites, can also be used as a germ cell mutagenicity test (29-31). However, in contrast to DNA fingerprinting, the tandem repeats are amplified by PCR using allele-specific PCR primers directed at the polymorphic sites in minisatellite regions. This allows the analysis of mutant alleles in individual gametes. Consequently this method can be used to map minisatellite length polymorphisms directly in the germ cell DNA (29-31). SP-PCR uses amplification of small aliquots of sperm DNA to screen thousands of sperm cells from an individual in one PCR reaction to detect changes in the repeat size of unstable minisatellite loci. Mutations detected by SP-PCR as products of altered length are counted and the rate of mutation expressed as a given number of events found in a given estimated number of sperm equivalents (32).

Quantitative SP-PCR methods were found to be appropriate for investigating the effects of chemotherapeutic mutagens and radiation on minisatellite mutation rates in human sperm (32-35). The advantage of this approach is that relatively small increases in mutation rate can be detected in individual samples, which makes it a sensitive method.
However, this technique also has several limitations that are predominantly related to interpretation of the data. First, this technique may potentially underestimate the true number of expansion events, since PCR is known to favour the amplification of the smaller allele. Second, the variants detected in this assay may be PCR artefacts rather than variants in repeat size. Third, SP-PCR is less precise in controlling for the number of cell equivalents analysed. Consequently, considerable care should be taken in accurately diluting and pipetting the DNA samples for SP-PCR in order to minimise variation, as the number of cells in each pool is the denominator in determining the rates of change. Furthermore, the efficiency of amplification of all the cell equivalents within a pool may be less than 100% (36).

In conclusion, SP-PCR seems less appropriate as a mutagenicity test than standard DNA fingerprinting of minisatellites and ESTRs. The disadvantages of this technique are mainly related to the use of PCR amplification, which causes misinterpretation of the results by PCR artefacts.

**Single-molecule PCR**

In addition to SP-PCR, single molecule PCR (SM-PCR) has been developed as a method for detecting new mutations in both the germline and somatic tissues (35). This method has been used to quantify the *in vivo* mutation frequency at the *Ms6-hm* locus in somatic and germ cells, and to determine whether mutation induction by environmental exposure and ionising radiation can be detected in pedigrees as well as directly in sperm DNA (35,37). This is an important addition to the pedigree approach for tandem repeat analysis, because mutations in sperm are not necessarily transferred to the offspring and, *vice-versa*, mutations in the offspring do not necessarily originate from the gametes, but can be induced during early embryogenesis. For this SM-PCR method, DNA samples are digested outside the ESTR array and distal to the PCR primer sites prior to amplification, to render genomic DNA fully soluble prior to dilution. Digested DNA is serially diluted and amplified with flanking primers (38). PCR products are analysed by electrophoresis followed by hybridisation with specific probes. The approximate number of amplifiable ESTR molecules in each dilution is estimated by Poisson analysis of the number of positive and negative reactions for ESTR-PCR products. Subsequently, a new set of PCR
reactions is seeded with each containing a mean of approximately one amplifiable ESTR molecule. PCR products are subsequently separated on an agarose gel, followed by hybridisation and scoring of the allele lengths. A band shift of at least 1 mm relative to the internal size marker indicates a mutation; smaller changes cannot be scored reliably (35).

SM-PCR has several advantages. First, experimental time might be reduced because mating and birth is unnecessary (it has been shown in laboratory mice that mutation rates in the offspring were indistinguishable from the rates in sperm DNA (35)). Second, SM-PCR was found to be useful for studying mutation induction at low dose exposures (35) and the majority of mutations were small changes in repeat copy number. These advantages allow a more thorough investigation of chemicals with different modes of action. Furthermore, using SM-PCR circumvents the requirement of sub-cloning. Yauk et al. (35) showed that SM-PCR can provide robust estimates of the frequency of ESTR mutations in somatic and sperm cells of irradiated and control male mice.

In addition to the limited number of in vivo results, in vitro studies have also shown that SM-PCR is a useful method for detecting mutations. Polyzos et al. (37,39) described the same method for the in vitro detection of de novo mutations in cultures of embryonic cells and noted that cells treated with ENU, B[a]P and etoposide showed a significant increase of the mutation frequency compared to untreated cells. They also indicated a similarity in mutation response between embryonic fibroblasts in culture, and somatic and germ cells in vivo at the ESTR locus (39).

In conclusion, SM-PCR methods are appropriate for the detection of germline mutations at the ESTR locus Ms6-hm. The reduction in time and costs make this method particularly suitable for screening large numbers of agents. A similar approach developed for minisatellites would be very useful for studying mutation frequencies in humans, but such methods are currently lacking.

Do differences between rodent and human spermatogenesis affect germline mutation induction?
The advantage of the methods described above is that they can be used to detect germline mutations in rodents and humans. This is essential because until recently extrapolation of
animal data was necessary for assessing potential genetic risks in the human population. Nonetheless, extrapolation might still be very useful in certain situations. Human exposure to genotoxic chemicals usually involves chronic exposure to low doses, making it much more difficult to study mutagenicity during spermatogenesis. In theory, this could lead to a misinterpretation of the effects due to the differences in spermatogenesis between rodents and humans (reviewed by Ehmcke et al. (40) and summarised in Figure 4). There are differences in type of stem cell and in mitotic expansion. Finally it must also be remembered that the vulnerability of germ cells towards genotoxins is known to alter during spermatogenesis due to cell turnover, differentiation and changes in DNA repair activity (41).

**Analysis of germline mutations: where do they come from and where do they take us?**

Both RAPD-PCR and the analysis of repetitive sequences might increase our understanding of how best to extrapolate from rodents to humans. However, important concerns are 1) the mechanisms through which the mutations arise are unknown, and 2) their biological relevance for the health of offspring is not clear.

_Mechanisms leading to mutations_

Alterations in RAPD-PCR profiles may originate from genomic alterations, DNA lesions that block the polymerase and secondary structures within the PCR product (13). Therefore, studying the origin of RAPD-PCR detectable mutations from a mechanistic viewpoint will be difficult, because multiple types of mutations can be involved. Gains or losses of tandem repeats are possibly easier to interpret, as the underlying mechanism might be the same. ESTR mutations have been observed after exposure to radiation but were also induced by chemical carcinogens that induce strand breaks or bulky DNA lesions (28,37,42). Although this non-specific detection of mutations induced by different types of DNA damage might be advantageous for the sensitivity of the assays, it complicates the mechanistic interpretation of the data. Therefore, more dedicated _in vitro_ and _in vivo_ experiments are needed to provide a definitive characterisation of the mechanistic basis for this type of mutation analyses. Moreover, these mutations may also
be the consequence of genomic destabilisation during early embryogenesis, which is supported by the observation that an increased number of mutations can arise in the maternal allele of the offspring of irradiated males and unexposed mothers (43). Therefore, mutations in both sperm and offspring should be observed so that the two processes can be differentiated. There is evidence that indirect mechanisms are involved in the induction of tandem repeat mutations; for instance recent findings indicate a potential role for epigenetic alterations (44). Since the epigenome can be transferred to subsequent generations, this mechanism might also be relevant for exposed populations.

Transcriptomics could contribute to this mechanistic understanding of germline mutagenesis by comparing gene expression in testes of exposed fathers. Despite the obvious difficulties in obtaining mRNA from human testes, gene-expression can be studied in mRNA obtained from ejaculates. The presence of mRNA in an ejaculate was discovered by Pessot et al. (45) and is thought to reflect gene expression during spermatogenesis (46). Therefore pathways involved in the induction of tandem repeat mutations may also be identifiable in humans. Studies in humans seem to be necessary to avoid problems with inter-species extrapolation. However, variability in the susceptibility towards genotoxic compounds may complicate the interpretation of the data and so controlled studies with laboratory animals are still required. Moreover, the use of genetically modified animals may allow further elucidation of mechanisms in germline mutagenesis.

**Biological significance of mutations detected**

It is almost impossible to predict the biological impact of germline mutations for offspring health. In principle, the RAPD-PCR assay can scan the entire genome including coding DNA regions, which can be identified by cloning and sequencing the altered PCR products (new PCR products, or PCR products that disappeared due to exposure). However, most of the mutations will probably be found in non-coding DNA. No studies to date have linked an increased mutational load, as assessed by RAPD-PCR, to an actual phenotypic alteration. Furthermore, it is still unclear whether tandem repeat mutations reflect effects in coding regions or provide a measure of other heritable genomic outcomes. Although the tandem repeat loci do not confer any observed phenotypes, many
tandem repeats are known to be related to transcribed genes and instability within these repetitive sequences has been shown to influence the function of these genes (47-50). For example, expansions within tandem repeat sequences are associated with approximately 20 well-characterised developmental and degenerative diseases (47). Nevertheless, mutations assessed by RAPD-PCR or tandem repeat analysis can be considered as biomarkers of adverse genetic events.

**Future perspectives**

The new approaches described are needed to test germline mutagenicity in humans exposed to environmental and dietary mutagens (51). Efforts need to be made to use new molecular techniques to overcome the limitations of classical tests with respect to time, costs and the number of animals needed for germline mutagenicity testing. Further these tests should fit in a broad range of applications; genomic alterations should be measurable in humans, animals and their offspring, in multiple organs, and at different stages during spermatogenesis.

Table II provides an overview of the advantages and disadvantages of RAPD-PCR and DNA fingerprinting of tandem repetitive DNA sequences compared to classical germ cell assays. The two new methods fulfil most of the demands, including their applicability in humans, but still require further technical validation. Too few experiments have been performed to confirm the suitability of these methods for detecting germline mutations. In addition, not enough is known about how tandem repeat mutations are formed, and the type of mutations analysed by RAPD-PCR. Therefore, the biological consequences of these mutations for the offspring are unknown. However, the improved sensitivity, which results in the use of small sample sizes and consequently lower costs, means that these methods are promising for the testing of low doses of mutagens and long-term exposure to mutagens on germline mutations and heritable effects.

DNA fingerprinting for repetitive sequences is the most promising new method for mutagenicity testing, while RAPD-PCR can be considered as a semi-quantitative screening method. Furthermore, DNA fingerprinting is a sensitive method for detecting germline mutations in sperm, and for establishing whether these mutations can be
transmitted to the progeny after long-term, low dose exposure to pollutants in experimental animals, free-living animals, and humans.
Funding
Cefic Long-Range Research Initiative (LRI) Innovative Science award 2004 (to R.G.);
References


**Table I.** Structural differences of tandem repetitive sequences. The structure of tandem repetitive sequences can be classified according to sequence size and number of repeats. Abbreviations: ESTRs, Expanded Simple Tandem Repeats; nt, nucleotides.

<table>
<thead>
<tr>
<th>Tandem repetitive sequences</th>
<th>Sequence size (nt)</th>
<th>Number of repeats</th>
<th>Total size</th>
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<tbody>
<tr>
<td>Microsatellites</td>
<td>2-5</td>
<td>10-100</td>
<td>-</td>
</tr>
<tr>
<td>Minisatellites</td>
<td>5-100</td>
<td>Up to 6000</td>
<td>Up to 30 kb</td>
</tr>
<tr>
<td>ESTRs</td>
<td>4-6</td>
<td>Up to 4000</td>
<td>Up to 16 kb</td>
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Table II. Comparison of the characteristics of germ cell assays. + indicates favourable, - indicates unfavourable

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>DNA fingerprinting</th>
<th>RAPD-PCR</th>
<th>Classical germ cell assays</th>
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<tr>
<td>Validation</td>
<td>-</td>
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<tr>
<td>Reproducibility</td>
<td>+</td>
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<td>Costs</td>
<td>+</td>
<td>++</td>
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<tr>
<td>Time</td>
<td>+</td>
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<tr>
<td>Number of animals required</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Sensitivity</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Applicable to humans</td>
<td>++</td>
<td>++</td>
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<td>Coding DNA</td>
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<tr>
<td>Phenotypic effects</td>
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<td>Defined mechanism of mutation</td>
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Figures

Fig. 1. A parallelogram approach to estimate the genetic risk in humans. Adapted from Adler (1996) (8).
Fig. 2. Simplified overview of the RAPD-PCR principle: A. a single random primer is able to bind at several sites in the DNA (indicated by arrows) and produces fragments of different lengths upon PCR (products 1 and 3), which can subsequently be detected after gel electrophoresis (panel on the right). B. if a mutation is introduced at a site at which a primer binds, this binding site is lost (indicated by *) under stringent conditions, and so the PCR product (product 1) cannot be formed. C. mutations can also lead to the introduction of new binding sites for the primer (indicated by *), leading to the formation of a new PCR product (product 2). Situations B and C can involve a range of genomic alterations such as deletions, insertions and chromosomal rearrangements. If the distance between two primers due to a genomic alteration is too large for effective amplification during PCR, the PCR product will also be lost. However, a deletion may result in primers that are close enough to allow amplification.
Fig. 3. Simplified overview of tandem repeat mutation analysis. Children always inherit one tandem repeat allele from their mother and one from their father. Changes in allele size indicate a deletion or insertion of one or more repeat units. A. Offspring 1 has inherited allele 1 from the mother and allele 2 from the father and no changes in allele size are detectable. There are also no changes detectable in offspring 2, who inherited another allele from the father. Offspring 3 has inherited allele 1 from the mother and allele 2 from the father. However, a change in repeat size of the allele inherited from the father (allele 2) has occurred, which indicates a deletion of one repeat unit. In offspring 4, a change in repeat size of allele 1 has occurred. A deletion of one repeat unit is visible in the allele inherited from the mother. B. Changes in allele size are visible after hybridisation of digested and electrophoresed DNA, which gives a pattern of tandem repeat fragments unique for a person.
**Fig. 4.** Schematic overview of spermatogenesis in mice and humans. This scheme shows the characteristics of different cell stages during spermatogenesis that are important for the formation of mutations in germ cells at different stages. Differences in spermatogenesis between mice and humans are predominantly at the level of stem/progenitor cells and the number of differentiating cells after mitotic expansion. Abbreviations: Prog, progenitor.