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Review article

## Multipoint linkage map from sperm typing data

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**Summary** — Sperm typing makes it possible to construct accurate multipoint linkage maps based on a theoretically unlimited number of meioses. The high sperm number from single individuals enables the dissection of hyper-recombinant regions and presents an ideal situation for studying recombination rate variability in mammals. Of particular relevance for livestock is the fact that fine structure mapping of functional genes by sperm typing can be used to identify the breakpoints between conserved syntenic groups in different species. This is important for the extrapolation of positional information from the highly developed human map to the lower density maps in farm animals.

### **sperm typing / multipoint map / livestock**

**Résumé** — La cartographie génétique multipoint à partir du typage des spermatozoïdes. Le typage des spermatozoïdes permet l'établissement de cartes génétiques multipoint précises à partir d'un nombre théoriquement illimité de méioses. Le grand nombre de spermatozoïdes provenant d'un individu permet la dissection des régions hyperrecombinantes et fournit un support idéal pour étudier la variabilité du taux de recombinaison chez les mammifères. D'un grand intérêt en élevage, la cartographie fine de gènes fonctionnels à partir du typage des spermatozoïdes, permet d'identifier les points de cassure entre des groupes de synténie conservés dans différentes espèces ; cela est important pour extrapoler l'information positionnelle de la carte humaine très élaborée à partir de celle des cartes moins denses des animaux domestiques.

### **typage des spermatozoïdes / carte génétique multipoint / bétail**

## **INTRODUCTION**

Higher eukaryotes including mammals exist predominantly as diploids and produce haploid gametes that normally do not have the capacity to undergo cell division. Construction of genetic maps in mammals has tra-

ditionally been performed by studies of the somatic DNA of the parents and offspring combined with appropriate statistical methods. In many situations, however, a limited number of families, few offspring per family and uncertain linkage phases for the loci under investigation make this method inac-

curate, especially when resolving genetic distances less than a few centimorgan (cM). Several years ago, following the development of the polymerase chain reaction (PCR) technique, a new approach to measuring genetic recombination between DNA markers without pedigree was proposed (Li et al, 1988). The method, termed *sperm typing*, depends on ultimate sensitivity, starting with one copy of DNA from a single sperm as a template for PCR. Each haploid sperm represents a meiotic event, and a direct count of recombinant and nonrecombinant meiotic products can be made and used to estimate recombination frequencies. An estimate of the frequency of recombination is obtained simply by dividing the number of recombinant sperm by the total number of sperm examined. As a potentially unlimited number of meioses can be analysed for each individual, the method is extremely useful for constructing a fine structure map of closely linked loci. Beside this obvious virtue, the exceptionally high sensitivity of the PCR method offers a unique opportunity to study previously inaccessible biological problems concerning the variation that occurs at the level of single cells. In this paper, the experimental problems relating sperm typing are discussed. Furthermore, the potential use of sperm typing for constructing multipoint linkage maps and studying basic genetics in farm animals is presented.

#### **EXPERIMENTAL PROBLEMS IN SPERM TYPING**

In most sperm typing experiments, several loci are amplified from one copy of DNA in hundreds or thousands of samples. The repetitious amplification of the same loci increase the chance of contamination and demand a laboratory especially organized for such work. Sperm typing involves three

major steps: i) sorting of individual sperm followed by lysis to release the DNA from the sperm head; ii) amplification of the polymorphic regions from one copy of DNA by PCR; and iii) analysis of the allelic state of the loci.

#### ***Sorting and lysis of single sperm cells***

In the original procedure of sperm typing (Li et al, 1988), single sperm samples were prepared manually using a micromanipulator and lysed through the addition of proteinase K, DTT and SDS. This procedure was inaccurate in the sense that a significant portion of the sperm samples contained none or more than one sperm, and inefficient due to an incomplete lysis of the sperm cells. Since then, a more efficient protocol has been developed including KOH and DTT (Li et al, 1991). The method of preparing single sperm by micromanipulation has been replaced by two methods: i) sorting single sperm by flow cytometry, and ii) manual isolation of sperm cells fixed in low melting point agarose. The use of flow cytometry enables 100 sperm to be sorted within a few minutes, which might increase the speed of processing tenfold vs micromanipulation (Goradia et al, 1991). Although the accuracy of preparing only one sperm by flow cytometry is much better than the micromanipulation protocol, some samples still contain none or more than one sperm. Another problem with flow cytometry is that the equipment required is very expensive and usually not found in most laboratories. Alternatively, individual sperm cells can be selected by fixing the sperm in low melting point agarose gels and manually picking them under an inverted phase microscope (Lien et al, 1993). The agarose method is very accurate but laborious as even experienced technicians can only pick up 100 sperm per hour.

### ***Amplification of the polymorphic regions from one copy of DNA***

Starting with only one DNA molecule as the template requires the ultimate sensitivity of the PCR technique. It is necessary to carry out a two-step PCR, with nested primers, to achieve acceptable efficiency in the analysis. Two alternative strategies have been suggested for sperm typing: i) use of specific primers in both PCR steps; and ii) perform an initial whole genome amplification prior to the PCR reactions with specific primers. The goal of whole genome amplification is to increase the number of the haploid genome copies without producing an unbiased number of loci. The method suggested for this purpose is primer extension preamplification (PEP), which involves multiple rounds of extension with a mixture of 15-base-long oligonucleotide primers (Zhang et al, 1992). Starting with a single haploid cell, 50 PEP cycles produce an estimated average of 60 copies, and at least 78% of the genome is represented at least 30 times (Zhang et al, 1992). Compared to using specific primers in the initial PCR step, PEP has a lower efficiency but the advantages of using it are that genotyping results can be confirmed and that the method allows for including new loci not present in the first analysis.

### ***Analysis of the allelic state of the loci***

The choice of method for determining the allelic state is highly dependent on the loci included in the analysis. In general, the best methods are applicable to multiplexing in PCR and allow for simple and simultaneous determination of the alleles at several loci.

### ***Source of errors in sperm typing***

Most applications of sperm typing involve the search for a few recombinant or mutated

sperm among hundreds or even thousands of samples analysed. The nature of such analyses implies particular concern about sources of errors such as: i) a sample tube may not contain any or may have more than one sperm; ii) the sperm sample may be contaminated with PCR products carried over from other PCR reactions; iii) misincorporation of errors by Taq polymerase; iv) one of the alleles at a given loci is amplified more efficiently than the other (allele-specific PCR); and v) the polymorphic regions of interest in the sperm sample may not be amplified to a detectable level due to low efficiency in the sperm lysis or PCR amplification. A mathematical approach has been developed to analyse sperm typing data that are potentially complicated by such errors. By means of a maximum likelihood procedure, most of the above factors are reestimated statistically and their standard errors and the recombination fraction are calculated (Cui et al, 1989).

## **MULTIPOINT LINKAGE MAP FROM SPERM TYPING DATA**

The recombination rate in higher mammals has traditionally been determined in reference families. This method efficiently orders markers along a chromosome, but has limited statistical power for accurately comparing low frequencies of recombination due to the limited sample sizes. Because of the unlimited number of meioses available, sperm typing has been shown to be very useful for gene ordering and for high-resolution recombination analysis of closely linked loci whose chromosomal localization has been only approximately defined by pedigree analysis (Lewin et al, 1992; Furlong et al, 1993; Lien et al, 1993).

### ***Use in comparative gene mapping***

Another important feature of sperm typing is the possibility of high resolution genetic

mapping of markers with a low polymorphic information content (PIC), such as many functional genes. Because of their specific function, these genes are conserved across species and form the backbone of comparative mapping strategies (O'Brien et al, 1993). Recent work in our laboratory shows that fine structure mapping of bovine genes by sperm typing can be used to narrow down the chromosomal breakpoints between cattle and humans and to further improve the resolution of synteny conservation between these two species. Among other applications, such comparative information might be particularly useful in the search for genes affecting quantitative trait loci (QTL) in cattle. Although livestock maps have improved dramatically during the last few years, they are still in a developmental stage compared to the human and mouse mapping efforts. It is reasonable to believe that the wealth of information already available in human and mouse maps could provide potential candidate genes for QTL with a chromosomal localization in cattle.

#### ***Genetic mapping of marker intervals with known physical distances***

The linear order of loci in sperm typing experiments has normally been inferred by carrying out discrete three-point crosses. The possibility of analysing multiple markers from thousands of meioses by sperm typing, however, also creates an ideal situation for dissecting marker intervals with much higher recombination frequencies than the genome average (hot spots) and for detecting double recombinants. The first multipoint linkage maps constructed from sperm typing data were reported by Schmitt et al (1994) who analysed 903 sperm cells for seven markers in the human pseudoautosomal region (PAR), and Hubert et al (1994), who analysed 602 sperm cells for seven markers in a hyper-

recombinant region close to the locus for Huntington disease on human chromosome 4. By accurately determining the genetic distance between markers, whose physical distance is exactly known, these studies were able to dissect hyper-recombinant regions in the genome. Evidence for double recombinants within a physically short distance were also detected. These results show that sperm typing has the potential to examine marker intervals at a high resolution and possibly reveal the molecular basis for hyper-recombination in mammals. Although hyper-recombinant regions of meiotic recombination have been well characterized at the DNA level in yeast, only a limited amount of information is known about them in higher eukaryotes.

#### ***Variability of recombination***

It is well known from experiments with laboratory animals (*Drosophila*, *Tribolium* and mouse) that the recombination is not a constant, but exhibits a considerable amount of variability and is affected by certain factors including age, sex, etc. Although there is some evidence for the presence of variability of the recombination rate in large mammals, the recombination fraction is modelled as a constant parameter, eventually within subgroups such as males and females. Multipoint mapping of loci from sperm typing data offers a unique opportunity for studying the variability of the recombination rate in mammals. The first sperm typing study revealing individual variability of recombination rate was undertaken by Park et al (1995) who analysed two bulls in a marker interval on bovine chromosome 23. The main objective of the experiment, however, was to extend the linkage and comparative maps of bovine chromosome 23 and the design was not optimal for studying recombination rate variability. Recently, we completed a sperm typing project to sys-

tematically study male recombination rate variability in cattle, focusing on between-individual and between-family variabilities. Questions were also considered concerning differences in variability depending on the length of the chromosome, the position of the interval on the chromosome, interference in recombination and differences between X- or Y-bearing sperm. In total, 2 214 single sperm cells from 37 bulls belonging to six families were typed for 11 loci on bovine chromosomes 6, 23 and the sex chromosomes. Individual variability in the recombination rate was detected for one interval on chromosome 23 and one interval in the PAR of the bovine sex chromosome. Highly significant variability between families was found for another marker interval in the PAR.

#### OTHER APPLICATIONS OF SPERM TYPING

The theoretically unlimited number of meioses to be analysed in the sperm typing approach presents an opportunity to study the stability of di- and trinucleotide repeats. It is also possible to estimate the male germline mutation rate for any allele size (repeat number) of a given marker in single individuals. Sperm typing has been utilized by Leeflang et al (1995) to study the frequencies of trinucleotide repeat mutations (CAG) in the Huntington disease gene in three affected and two normal individuals. Disease alleles (38–51 repeats) showed a very high mutation frequency (92–99%), whereas normal alleles with 15–18 repeats and 30 repeats showed mutation rates of 0.6 and 11%, respectively. Two studies by Zhang et al (1994, 1995) used a similar approach when examining the frequency of trinucleotide repeat mutations on the X-linked androgen receptor and the gene causing Kennedy disease. The overall aim of these studies was to achieve a better

understanding of the expansion and contraction processes of simple tandem repeats (STR).

The unlimited number of sperm from single individuals also makes it possible to estimate the frequency of sequences that have been created by interallelic gene conversion in the germline. Detection of such inter-locus or inter-allelic segmental exchanges by sperm typing was demonstrated by Zangenberg et al (1995) studying the role of interallelic gene conversion in generating the extensive allelic diversity at the major histocompatibility complex (HLA) in humans.

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