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A New Source of Polymorphic DNA Markers for Sperm Typing: Analysis of Microsatellite Repeats in Single Cells

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Summary

We show that dinucleotide and tetranucleotide repeat polymorphisms can be analyzed in single cells without using radioactivity or denaturing gels. This provides a rich new source of DNA polymorphisms for genetic mapping by sperm typing. The recombination fraction between two CA repeat polymorphisms was determined after whole genome amplification of single sperm, followed by typing of two different aliquots, one aliquot for each polymorphic locus. Single-cell analysis of microsatellites may also be valuable both for preimplantation genetic disease diagnosis based on single-blastomere or polar-body analysis and for the typing of forensic or ancient DNA samples containing very small amounts of nucleic acid.

Introduction

Sperm typing (Li et al. 1988; Arnheim et al. 1990) measures the frequency of genetic recombination between DNA polymorphims by determining the genotype of individual sperm cells by using PCR (Saiki et al. 1985, 1988; Mullis and Faloona 1987). Recombination fractions estimated by sperm typing have been shown to be consistent with data from family studies (Cui et al. 1989). Because there is a virtually unlimited number of sperm available, recombination can be measured at a resolution impossible to achieve with pedigree analysis. Sperm typing is also useful in the case of polymorphisms that have very low informativeness, and, consequently, insufficient numbers of families are available to allow an accurate estimate of the recombination fraction. Typing a large number of sperm from a single informative individual makes it possible to estimate the recombination fraction (Hubert et al. 1992). Sperm typing has also been used to order linked loci in three-point crosses (Goradia et al. 1991).

While sperm typing has been demonstrated to be a practical and rapid method of measuring recombination, its application heretofore has been limited by the lack of base-substitution polymorphisms where DNA sequence information is available for PCR analysis. Considerable effort is required for the conversion of RFLPs to a PCR format (see Goradia et al. 1991).

Several years ago, the lengths of microsatellite repeat tracts in the human genome were found to be highly polymorphic (Litt and Luty 1989; Weber and May 1989). A significant effort has been made to characterize these polymorphisms for application to genetic mapping using pedigree analysis. Microsatellite repeat tracts are quite short, and allele typing was originally based on electrophoretic analysis of radioactive PCR products made by using primers that immediately flank the repeated sequence. Accurate typing of alleles that differ by as little as 2 bp can be achieved using DNA sequencing gels beginning with nanogram amounts of genomic DNA.

A common feature of microsatellite repeat typing is that the PCR product of each allele consists not of a single unique band but of several closely spaced fragments due, possibly, to the slippage of the *Taq* polymerase while replicating the repeating unit. Dinucleotide repeat polymorphisms would be especially valuable for sperm typing studies, but we were initially concerned that the large number of PCR cycles required to amplify a single-copy gene, starting from a single sperm cell, would only exacerbate the slippage problem and make it impossible to distinguish among

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the alleles. We show, however, that, with the correct PCR conditions, allelic typing of dinucleotide (CA) and tetranucleotide (ATTT) repeat polymorphisms in single cells is accurate and efficient and can even be carried out without the use of radioactivity or DNA sequencing gels.

We specifically applied sperm typing to measuring the recombination fraction between two chromosome 19 CA repeat polymorphisms. In these experiments we applied a whole-genome amplification procedure called "*p*rimer *e*xtension *p*reamplification" (PEP) (Zhang et al. 1992) to each sperm before typing. This allows at least 24 independent aliquots to be tested from each individual sperm. In our experiments two aliquots were used, one for each CA repeat polymorphism, and, in some cases, additional aliquots were studied to confirm the sperm's genotype.

Material and Methods

Screening Semen Samples for Microsatellite Heterozygotes

Individuals heterozygous at APOC2, D19S49, and D9S52 were found by screening samples from a semen bank. The former two loci are polymorphic for a CA tract, as reported by Weber and May (1989) and Weber et al. (1990), respectively, while the latter is polymorphic for a tetranucleotide repeat (ATTT; Wilkie et al. 1992). One microliter of semen from each donor was lysed and neutralized according to published procedures (Cui et al. 1989; Li et al. 1991). Two microliters of a 1:100 or 1:1,000 dilution (depending on the semen sample) of lysed and neutralized sample was amplified in two rounds of PCR (Li et al. 1990). The PCR reaction mix contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 1 unit *Taq* DNA polymerase, and 100 μM of each dNTP in a final volume of 50 μl. An MJResearch thermocycler that accomodates microtiter plates was used for amplifications.

First-Round PCR

We used three pairs of outside primers, each pair flanking one of the three loci (fig. 1). All the primers were present in each reaction mixture. Twenty-five cycles were carried out: 10 cycles of 92°C for 30 s, for denaturation, and 60°C for 4 min, for annealing and extension, followed by 15 cycles of 92°C for 30 s, for denaturation, and 60°C for 3 min, for annealing and extension. The final cycle was followed by a 5-min extension at 72°C. The primer concentrations are shown in table 1.

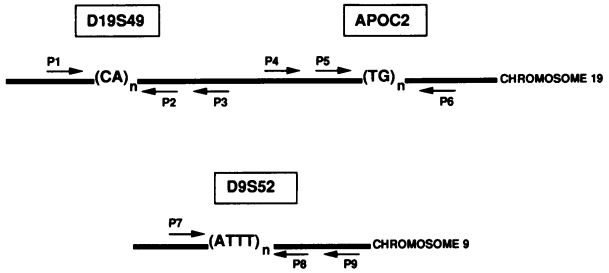

Figure 1 Position of PCR primers for D19S49, APOC2, and D9S52. The outside primer pairs are 1 and 3, 4 and 6, and 7 and 9 for the three loci, respectively. The remaining primer at each locus is used for heminesting in the second round. The bracketed sequence represents the repeating unit.

Second-Round PCR

Two microliters of first-round product were amplified in three separate second-round reactions (one for each of the three loci), by using one of the outside primers and an internal primer (e.g., P1 and P2 for D19S49; fig. 1). This typing procedure will yield PCR products that differ in length according to the number of microsatellite repeating units that are characteristic of each allele. Except for different primer concentrations (table 1), the reaction components for this second round were as in the first round. Twenty cycles were carried out (92°C for 30 s, for denaturation, and 60°C for 1 min, for annealing and extension). The final cycle ended with a 5-min extension at 72°C.

PCR Conditions for Single-Sperm Analysis

Single sperm from a triply heterozygous individual were isolated by fluoresence-activated cell sorting (Li et al. 1991) and were deposited into 96-well Falcon microtiter plates containing 5 μl of lysis solution. After an 8-min incubation at 65°C, 5 μl of neutralization solution was added. All 10 μl of lysed sperm solution were used for amplification. The number of rounds, PCR conditions, reaction components, and volumes were the same as described for semen screening, except that KCl was left out of the first-round PCR buffer (Li et al. 1991; the potassium is supplied by the sperm lysis solution). Six microliters of PCR product from each locus-specific second-round amplification were mixed, 3 μl of loading dye were added, and electrophoresis was carried out on a 15-cm × 19-cm × 1.5-mm-thick 8% acrylamide gel at 200 V for 2.5 h. Genotypes were determined after staining with ethidium bromide.

Table 1

PCR Primers and Primer Concentrations for First and Second Round of Amplification

Locus	Primer		Sequence (5′ to 3′)	Primer Concentration (μM)	
	Number	Lab Code		First Round	Second Round
APOC2	P4	A196	GGGAGAGGGCAAAGATCGATAAAGCAGGAA	.2	. . .
APOC2	P6	A195	GCCTGGGCTACATAGCGAGACTCCATCTCC	.2	.9
APOC2	P5	A197	GGCACAATATTAGAAGCCCGTGTTGGAACC	. . .	.9
D19S49	P3	O019	AAAGTGCAGGGATTAGAGGCGTGAGCTACC	.2	. . .
D19S49	P1	O018	GAAGGTGACAGTTCCTCAGGCCCACAGTAA	.2	.9
D19S49	P2	O020	GCCCCTAGGGTTTTAGATTGAGTGTTGTTGACC	. . .	.9
D9S52	P9	A201	TTAGCCAGGTGTTGTGGTGG	.2	. . .
D9S52	P7	A200	CATCTAGCTAGAGGGAGGTT	.2	.9
D9S52	P8	A202	GGGAGGCAGAGGTTATAGTG	. . .	.9

Estimating the Recombination Fraction between D19S49 and APOC2

Individual sperm from another donor informative for D19S49 and APOC2 were isolated and lysed as described above. We used a new procedure, PEP (Zhang et al. 1992), to enhance the success of genotype determination in each sperm. Each lysed and neutralized sperm was first subjected to a pretreatment of 50 primer extension cycles by using a mixture of 4^{15} different 15-base-long oligonucleotides, as described by Zhang et al. (1992), with the slight modification that the random primer was at a concentration of 40 μM and that the final volume was 50 μl. This procedure increases the number of copies of a unique-sequence gene, from 1 to a minimum of 30 copies. Two 5-μl aliquots were taken from the PEP reaction; one was analyzed for D19S49 and the other for APOC2, in the standard two-round protocol described above for semen analysis, with the following exceptions: the APOC2 primers were each at 0.4 μM for the first round and at 1.2 μM for the second round. In addition, 10 (rather than 15) cycles were carried out with 3-min annealing and extension times in the first round. Finally, 21 (rather than 20) cycles were used in the second round. Electrophoresis was carried out on a 15-μl sample independently for each locus, on 15-cM × 25-cm × 4-mm 3% Nusieve, 1% regular agarose gel. Three 20-well combs were set at 8-cm intervals along the length of the gel. Electrophoresis was at 120 V for 2 h. Gels were stained with ethidium bromide.

Results

Microsatellite Analysis in Single Sperm

In preliminary experiments we tested whether we could amplify microsatellite repeat polymorphisms by beginning with a single cell. The amplification products from 14 single sperm from a donor triply heterozygous at APOC2, D19S49, and D9S52 are shown in figure 2. On the basis both of the sizes of the APOC2 PCR products (101 and 109 bp) and of the already determined sizes of the known APOC2 alleles, our data suggest that the alleles of our donor contain 18 and 22 dinucleotide repeats, respectively. The sizes of the D19S49 products (120 and 132 bp) indicate that the number of dinucleotide repeats in the two alleles are, respectively, 15 and 21. PCR products from the two alleles at D9S52 are 152 and 156 bp (12 or 13 repeats).

The ability to amplify the single target molecule present in the sperm so that it can be detected by ethidium bromide staining in the absence of significant background bands results from using two rounds of PCR, with a heminesting primer in the second round (Li et al. 1990). This heminesting step is critical for background reduction, since the absolutely large number of PCR cycles required for single-molecule detection can lead to the formation of nonspecific PCR products when only the two original primers are used for amplification.

We also found that the number of extra DNA fragments usually characteristic of each APOC2 and

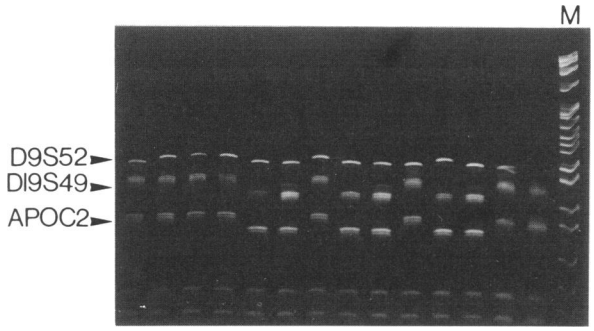

Figure 2 PAGE of PCR products from the individual triply heterozygous at D19S49, APOC2, and D9S52. Each lane contains the PCR products from the three loci, amplified in a single sperm, except for lane M, which is pBR322 cut with *Msp*I. Not unexpectedly, the intensity of the band characteristic of any one allele may vary slightly from sample to sample.

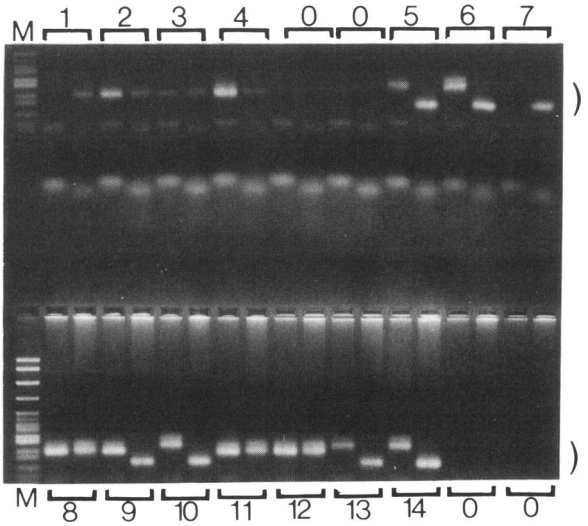

Figure 3 Agarose gel electrophoresis of PCR products from 14 sperm obtained from the second individual heterozygous at D19S49 and APOC2. The whole genome of each sperm was first amplified using PEP. Two aliquots were taken, one analyzed for D19S49 and the other for APOC2. The sizes of the alleles from the two loci overlapped (one allele from D19S49 differed only by a single base pair from one of the APOC2 alleles), and therefore the amplification products from the different loci were loaded in adjacent lanes, D19S49 in the first and APOC2 in the second. These pairs of lanes are numbered 1–14. Lanes 0 represent controls with no DNA added. The position of migration of the specific amplified products is marked by an end parenthesis to the right of the lanes.

D19S49 allele were reduced significantly if we carefully optimized the number of PCR cycles used in both the first and second rounds (data not shown). As a consequence, it was easy to distinguish between the alleles at each locus.

Recombination between D19S49 and APOC2

We examined a total of 120 sperm at D19S49 and APOC2 from a second doubly heterozygous sperm donor. Two aliquots were taken from each PEP-treated sperm, one for D19S49 and one for APOC2. In this individual the allele sizes for D19S49 were 136 bp (23 repeats) and 122 bp (16 repeats). For APOC2 they were 123 and 101 bp (29 and 18 repeats, respectively). For rapid typing of these alleles we used Nusieve agarose rather than acrylamide gels. Typical data are shown in figure 3 and are summarized in table 2. The two most common (nonrecombinant) sperm genotypes were Ab and aB and establish the phase as Ab/aB (see note to table 2). Among the 120 total sperm, 9 were recombinant. Since each sperm was treated with the PEP procedure, we were able to take another aliquot from all apparent recombinant sperm to confirm the genotype. Because PEP and PCR are not 100% efficient, some samples may not be amplified to a detectable level, and bands may be missing (e.g., see fig. 3, sample 4). To estimate the recombination fraction (θ), we used a statistical method that takes into consideration (1) any possible between-allele differences in the probability of being amplified to a detectable level, (2) contamination rates, and (3) the number of sperm actually sorted into each microtiter

Table 2

Typing Results from Single Sperm Treated with the PEP Procedure

Observed Sperm Type	No. of Sperm
A--B	43
-aB-	53
A-B-	4
-a-b	5
A---	2
-a--	5
--B-	2
---b	3
Aa-b	1
A-Bb	1
----	1
Total	120

NOTE.—The individual was heterozygous at D19S49 and APOC2. We call the 136- and 122-bp PCR products "allele A" and "allele a," respectively, for D19S49, and we call the 123- and 101-bp PCR products "allele B" and "allele b," respectively, for APOC2.

Table 3

Results of the Maximum-Likelihood Analysis of the Sperm Typing Data from Table 2

Parameter[a]	Estimate	Standard Error	95% CI
θ	.0831	.0269	.0305–.1357
Efficiency:			
A	.9373	.0346	.8694–1.0000
a	.9695	.0271	.9164–1.0000
B	.9160	.0395	.8390–.9934
b	.9634	.0294	.9059–1.0000
Contamination:			
A	.0000	Not available	Not available
a	.0199	.0187	.0000–.0564
B	.0189	.0180	.0000–.0542
b	.0000	Not available	Not available
No sperm	.0062	.0088	.0000–.0235
One sperm	.9938	.0088	.9764–1.0000
Two sperm	.0000	Not available	Not available

NOTE.—Estimates of each parameter and its standard error and 95% CI are given for the general model, which has a maximum log likelihood value of −350.84.

[a] A and a are the D19S49 alleles, and B and b are the APOC2 alleles defined in table 2.

well (Cui et al. 1989; Goradia et al. 1991). The results of this analysis are shown in table 3. The θ value for these two loci was estimated to be .083 (95% confidence interval [CI] = .030–.136). The results are virtually the same if the obvious error categories in table 2 (all but AB, Ab, aB, and ab) are ignored and if θ is calculated by dividing the nine recombinants by 105 sperm (θ = .083; 95% CI = .029–.140). Because of PEP, each sample from a single sperm should have contained several rather than a single target molecule, and, as a consequence, our efficiency of amplification is quite high (table 3).

Discussion

The ability to analyze microsatellite repeat polymorphisms on samples with only one or a few target molecules has implications for genetic mapping using sperm typing, preimplantation genetic disease diagnosis, forensics, and the analysis of ancient DNA samples. Our microsatellite typing procedure does not require the use of radioactivity or DNA sequencing gels. Recent reports (Love et al. 1990; Oudet et al. 1991) have shown that much larger amounts (100–200 ng) of genomic DNA can also be typed for CA repeats, without using radioactivity or DNA sequencing gels. We anticipate that our particular set of PCR conditions for single-cell analysis will be readily adaptable to other microsatellite polymorphisms, without much modification.

Using nondenaturing acrylamide gels, our typing protocol is capable of distinguishing between alleles that differ by two or more dinucleotide repeats. The effect that this would have on finding individuals informative for CA repeat polymorphisms in sperm-typing experiments can be calculated. On the basis of the analysis of 87 marker loci in each of 40–80 unrelated Caucasian individuals, average heterozygosity

$$1 - \sum_{i=1}^{n} p_i^2 ,$$

where p_i is the frequency of the ith allele among n alleles) was found to be 67%. If one can only distinguish between alleles that differ by two or more CA repeats or 4 bp, then the heterozygosity

$$\sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2p_i p_j ,$$

where the size of the ith allele minus the size of the jth allele is ≥ 4) is, on average, 43%. The effect that this lower heterozygosity has on sperm typing is not significant, since one only has to look at a few more sperm donors (1.5 times as many) before finding one that is informative for the locus of interest. Once the individual is found, of course, a virtually unlimited number of meiotic products can be examined.

We estimated θ between APOC2 and D19S49 as being .083 (95% CI = .030–.136). The reliability of our sperm-typing data is supported by the fact that our estimate overlaps the male recombination rate determined by typing the 40 CEPH reference families. A LOD score of 28.7 with θ = .342 for females and θ = .059 for males was observed by using LINKAGE (J. L. Weber, unpublished data). Results obtained with CRIMAP were very similar.

Being able to type microsatellite repeat polymorphisms could have a significant impact on the use of sperm typing for studying recombination. A major effort is being made to sequence the flanking regions of CA and other microsatellite repeats so that PCR typing can be used in pedigree analysis. The fact that these polymorphisms can now be typed using single sperm makes it likely that single-gamete analysis can be applied to many additional studies of genetic recombination. For example, multipoint mapping using

single sperm cells is now feasible because of the large number of potentially informative sperm donors available and because of the recent capacity to analyze at least 24 loci in each sperm by using PEP (Zhang et al. 1992). A linkage map made by sperm typing would not necessarily reflect the θ in female meiosis, but the order of the genes along the chromosomes would be identical. Another important advantage of the PEP procedure is that the genotype of any one sperm can be confirmed simply by analyzing additional flanking markers by using another aliquot from that PEP-treated sperm. This could be especially valuable for confirming rare recombinants.

The ability to type microsatellite repeat polymorphisms by beginning with very small DNA samples has a number of additional implications. For example, preimplantation genetic disease diagnosis can be carried out either on a single cell taken from an early embryo fertilized in vitro (Coutelle et al. 1989; Handyside et al. 1989, 1990; Holding and Monk 1989; Bradbury et al. 1990; Gomez et al. 1990) or on a polar body dissected from an egg before in vitro fertilization (Monk and Holding 1990; Strom et al. 1990). The ability to type microsatellite repeat polymorphisms in a single diploid cell would allow a diagnosis to be made if such a polymorphism were found to be tightly linked to a genetic disease. If PEP is not used, then the number of loci that could be typed from a single cell would be limited, and the genotype of a locus could not be confirmed. If PEP is used, the following factor needs to be considered: If the aliquot taken from the PEP reaction is small, then it may contain, by chance, a biased representation of the two alleles originally present in the cell, and one allele may even be missing. In such cases the analyses of multiple aliquots from the same sample are warranted (Navidi et al. 1992). The data on multiple aliquots can be used to calculate the probability that the sample is typed correctly.

The accurate typing of ancient DNA samples or forensics specimens for microsatellite repeats is now also possible when only very small amounts of DNA are available. Because these samples will initially consist of only a very small number of DNA fragments, the sampling problem must also be considered, regardless of whether PEP is used. However, using PEP would be advantageous, since more loci could be examined and since multiple aliquots could be taken from the same specimen, thus allowing more accurate typing (Navidi et al. 1992).

Acknowledgments

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