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Whole genome amplification from a single cell: Implications for genetic analysis

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ABSTRACT **We have developed an *in vitro* method for amplifying a large fraction of the DNA sequences present in a single haploid cell by repeated primer extensions using a mixture of 15-base random oligonucleotides. We studied 12 genetic loci and estimate that the probability of amplifying any sequence in the genome to a minimum of 30 copies is not less than 0.78 (95% confidence). Whole genome amplification beginning with a single cell, or other samples with very small amounts of DNA, has significant implications for multipoint mapping by sperm or oocyte typing and possibly for genetic disease diagnosis, forensics, and the analysis of ancient DNA samples.**

The sensitivity of the polymerase chain reaction (PCR; refs. 1–3) is great enough to allow the analysis of DNA in a single cell (4, 5). This led to the development of preimplantation genetic disease diagnosis using single cells from early embryos or polar bodies (6–11) and genetic recombination analysis using a single sperm (12–15) or oocyte (16). In all these cases the single cell can be analyzed only once and independent confirmation of the genotype of any one cell is impossible. We have developed a method to circumvent this limitation. Multiple copies of the DNA sequences present in a single cell are made by an *in vitro* method that we call *p*rimer-*e*xtension *p*reamplification (PEP). Multiple rounds of extension with the *Taq* DNA polymerase and a random mixture of 15-base oligonucleotides as primers produce multiple copies of the DNA sequences originally present in the sample. It is estimated that at least 78% of the genomic sequences in a single human haploid cell can be copied no less than 30 times. As a result, only a small aliquot of the amplified sample has to be used to analyze any one gene and material remains for additional analyses. Our method not only extends the possible applications of single cell studies but also has implications for the analysis of any small DNA sample.

MATERIALS AND METHODS

PEP of Single-Sperm DNA. The DNA sequences in individual sperm cells were copied by multiple rounds of primer extension using a collection of 15-base oligonucleotides in which any one of the four possible bases could be present at each position. Theoretically, the primer was composed of a mixture of 4^{15} (1×10^9) sequences.

Single human sperm were sorted by flow cytometry into 96-well Falcon microtiter dishes containing 5 μl of an alkaline lysis solution (200 mM KOH/50 mM dithiothreitol) as described (17, 18). After a 10-min incubation at 65°C, 5 μl of neutralization solution (900 mM Tris·HCl, pH 8.3/300 mM KCl/200 mM HCl) was added. To the lysed and neutralized sample was added 5 μl of a 400 μM solution of random primers (Operon Technologies, Alameda, CA), 6 μl of 10× K^+ free PCR buffer [25 mM $MgCl_2$/gelatin (1 mg/ml)/100 mM Tris·HCl, pH 8.3], 3 μl of a mixture of the 4 dNTPs (each at 2 mM), and 1 μl of *Taq* polymerase (Perkin–Elmer/Cetus, 5 units), and brought to 60 μl with water. Fifty primer-extension cycles were carried out in a MJ Research thermocycler (Cambridge, MA). Each cycle consisted of a 1-min denaturation step at 92°C, a 2-min annealing step at 37°C, a programmed ramping step of 10 sec/degree to 55°C, and a 4-min incubation at 55°C for polymerase extension. Each sample was then divided into aliquots and analyzed for specific DNA sequences.

Specific Gene Analysis. We tested the aliquots from a single sperm subjected to PEP for the presence of specific DNA sequences by using conditions that are capable of detecting single DNA molecules. A total of 12 loci were studied. We used a hemi-nesting strategy that enhances yield and specificity when starting with one target DNA molecule and allows the product to be detected by ethidium bromide staining (17, 18). The first round of PCR utilizes a pair of primers that flank the target sequence. The second round uses one of the two original primers and a second internal primer. In every case we took 2 μl of first-round product for the second round of PCR.

The PCR conditions and primer sequences for seven loci are described in ref. 18 (*PTH, LDLR,* and *HBG2*), ref. 13 (*D3S2, D3S11,* and *D3S12*), and ref. 14 (*D3S3*) with the modification that all four dNTPs (each at 100 μM) were used in the second round. Three microsatellite repeat polymorphisms (*D9S52, APOC2,* and *D19S49*) were analyzed (R.H., J. L. Weber, and N.A., unpublished data). The X chromosome-linked *STS* locus and the Y chromosome-linked *STS* pseudogene locus are amplified in the first round with the same set of primers (5′-GAGTGAAACTCACTCAGCAC-3′ at 0.1 μM and 5′-CCTTAGGAACCAGGAGATAC-3′ at 0.1 μM) at 92°C for 30 sec and 60°C for 4 min (11 cycles) or 3 min (next 31 cycles). Standard PCR buffer was used with all four dNTPs (each at 100 μM). The second round of amplification [92°C for 30 sec, 65°C for 1 min, and 72°C for 30 sec for 26 cycles; all four dNTPs (each at 8 μM)] included the first primer above and a mixture of two primers. One (5′-ACCGTACTTGCATGAGAAGCTGTCCCAAAGGA-3′ at 0.5 μM) is specific for the Y chromosome-linked pseudogene and the other (5′-TGGGAGACTGTCCCGAAGGT-3′ at 2 μM) is specific for the X chromosome-linked gene. Because the gene-specific primers differ in length, the size of the PCR products for each locus is also different. The primers for the DNA segment 25 kilobases distal to the pseudoautosomal boundary (pa) are 5′-GGAGTAAGACGCCATCTCAA-3′ and 5′-GATGTCGGCAAACTAGAACC-3′, and each is used at 0.2 μM. Only one round of a PCR using the conditions for the first round of *STS* amplification is required.

RESULTS

Our first experiment was designed to estimate the efficiency of the PEP procedure. Twelve single sperm were sorted by flow cytometry, lysed, and subjected to PEP for 50 primer-

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Abbreviation: PEP, primer-extension preamplification.

extension cycles using the mixture of random primers. After PEP, each sperm sample was divided into 30 aliquots that contained 2 μl of the original 60-μl PEP reaction mixture. For each sperm all 30 aliquots were tested for the presence of 1 of 12 specific DNA sequences by using PCR methods shown to be capable of detecting a single DNA molecule (17). Representative data for one of the sperm amplified by PEP and tested for the *PTH* locus are shown in Fig. 1. The controls in lanes 1–4 show that no specific gene product is produced if no DNA is added to the PEP reaction mixture. The 4 samples in lanes 5–8 come from a single sperm that was not subjected to PEP but was divided into 5 aliquots after lysis. Only 1 sample gave the specific *PTH* product. All but 1 of the 30 samples that received PEP aliquots were positive for *PTH*. The fraction of the 30 aliquots that were positive for the gene tested in each of the 12 sperm is shown in Table 1.

We can estimate a lower bound for the number of copies of each specific DNA segment present after PEP by using these data. In the worst case shown in Table 1, only 24 of the 30 aliquots were positive (*APOC2*). If the PEP product of this sperm had less than 30 copies of the *APOC2* DNA segment, then the chance that 24 or more of the 30 aliquots would be positive is less than 0.005. Therefore, we can assert with 99.5% confidence that at least 30 copies were present in this PEP reaction mixture. We are virtually certain that in the remaining 11 samples there also must have been at least 30 and probably more copies of the amplified sequence. The fact that the likelihood of detecting a single molecule without PEP can range from 72 to 96% (12–14) could contribute to underestimating the actual copy number.

The above experiment fails to show explicitly that the products of all 12 loci could have been detected in a single sperm. To test this possibility, 18 additional single sperm from the same donor were subjected to PEP. Twelve aliquots, each comprising 1/24th (2.5 μl) of the total PEP product, were taken for each sperm, and each aliquot was examined for a different one of the 12 loci. The success with which each of the 12 loci were detected in each of the 18 sperm tested is given in Table 2. Fig. 2 shows the results from one of the sperm.

We can also use these data to estimate the average number of copies per DNA segment produced by PEP. Of a total of

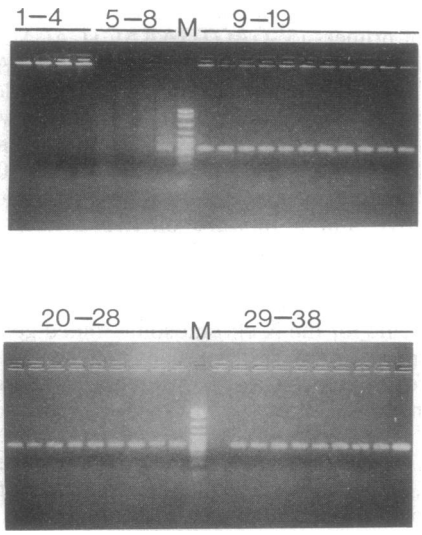

FIG. 1. Testing for the presence of *PTH* sequences in DNA from a single sperm subjected to PEP. Lanes: 1–4, *PTH* amplification of aliquots from a PEP sample to which no DNA was added; 5–8, amplification of *PTH* sequences in 4 aliquots of a lysed single sperm not subjected to PEP; 9–38, *PTH* amplification of 30 aliquots from a single sperm subjected to PEP; M, *Msp* I digest of pBR322 as molecular size markers.

Table 1. Analysis of PEP products from 12 sperm

Sperm	DNA segment	No. positive signals in 30 aliquots
1	*PTH*	29
2	*D9S52*	30
3	*D19S49*	30
4	*APOC2*	24
5	*D3S2*	30
6	*D3S3*	28
7	*D3S11*	30
8	*D3S12*	30
9	*HBG2*	30
10	*LDLR*	27
11	*STS*	30
12	pa	29

216 aliquots (12 × 18), 16 or 7.4% failed to give a positive signal. If the number of copies in a 2.5-μl aliquot is a Poisson random variable with an expected value of λ, then the maximum likelihood estimate of λ is 2.60. Since each PEP sample contained 60 μl, the average total number of copies is estimated to be 62.46 with a 95% confidence interval of 53.0–78.2. The results from the first experiment are not suitable for such a calculation since the 30 trials for any one locus are not independent.

The sperm donor used in this experiment was heterozygous at the microsatellite containing locus *APOC2* on chromosome 19 and, as a male, had the *STS* gene on the X chromosome and the *STS* pseudogene on the Y chromosome. The PCR systems for these loci were designed so that the allelic state at *APOC2* (the number of CA repeats) and the presence of the X or Y chromosome could be determined from the size of the PCR product alone. Among the 18 sperm, 9 contained one *APOC2* allele and 9 contained the other. Similarly, among the 17 samples that were positive for *STS*, 9 carried the X chromosome and 8 carried the Y chromosome. Fig. 3 shows the genotype of 9 of the 18 sperm. The segregation pattern of the *APOC2* alleles and the X and Y chromosomes can be clearly seen. The independent assortment of the sex chromosomes from chromosome 19 is also observed.

Our data can be used to estimate what fraction of the genome in a single cell can be expected to be amplified by PEP. It is likely that not all of the DNA sequences in a cell are capable of being amplified by a PCR just as not all sequences are capable of being cloned by conventional methods. Our estimate is made by using PCR systems designed from 12 cloned and sequenced genomic DNA segments. Therefore, it is more accurate to say that we are estimating what fraction of all the genomic sequences in a single cell capable of being cloned and amplified by a PCR can be amplifed by PEP. To make this estimate, we assume that the fraction of the genome that is amplified is approximately the same for each individual sperm. The data from the second experiment support this assumption. Of 216 amplification attempts (12 loci × 18 sperm), 200 amplifications were positive. Under the assumption above and further by assuming that each DNA segment that can be cloned and amplified by a PCR is equally likely to be amplified by PEP, the number of positive loci for any given sperm is a binomially distributed random variable with a probability of success equal to approximately 0.9259 (200 of 216). The actual frequency distribution of positive amplifications among the 18 sperm is consistent with that expected from 18 independent random variables with the binomial distribution described above (the Kolmogorov–Smirnov statistic d is 0.057, $P > 0.2$; see ref. 19). Since the PEP procedure appears to amplify approximately the same fraction of the whole genome in each sperm, we can treat the 12 loci amplified in the first experiment as if

Table 2. Analysis of 12 loci in 18 sperm

Sperm	Locus												Total positive loci, no.
	PTH	*D9S52*	*D19S49*	*APOC2*	*D3S2*	*D3S3*	*D3S11*	*D3S12*	*HBG2*	*LDLR*	*STS*	pa	
1	+	+	−	+	+	+	+	+	+	+	+	+	11
2	+	+	+	+	+	+	+	+	+	+	+	+	12
3	+	+	+	+	−	+	+	−	+	+	+	+	10
4	+	+	+	+	+	+	−	−	+	+	+	+	10
5	−	+	+	+	+	+	−	+	+	+	−	+	9
6	+	+	+	+	+	+	+	+	+	+	+	+	12
7	+	+	+	+	+	+	+	+	+	+	+	+	12
8	+	+	+	+	−	+	+	+	+	+	+	+	11
9	+	+	+	+	−	+	−	+	+	+	+	+	10
10	+	−	+	+	+	+	+	+	+	+	+	+	11
11	+	+	+	+	+	−	+	+	−	+	+	+	10
12	+	+	+	+	+	+	+	+	+	+	+	+	12
13	+	+	+	+	+	+	+	−	+	+	+	+	11
14	−	+	+	+	+	+	+	+	+	+	+	+	11
15	+	+	+	+	+	+	+	+	+	+	+	+	12
16	+	+	+	+	+	+	+	+	+	+	+	+	12
17	+	+	+	+	+	+	+	+	+	+	+	+	12
18	+	+	+	+	+	+	+	+	+	+	+	+	12
Total no. positive sperm	16	17	17	18	15	17	15	15	17	18	17	18	

they were chosen at random from among all the DNA sequences present in a single sperm. Since we are virtually certain that all 12 loci were amplified to at least a level of 30 copies (from the first experiment), we calculate with 95% confidence that the probability P of amplifying any sequence in the genome to a minimum level of 30 copies is no less than 0.78 ($P^{12} \leq 0.05$). Given the nature of our assumptions, this is a conservative estimate.

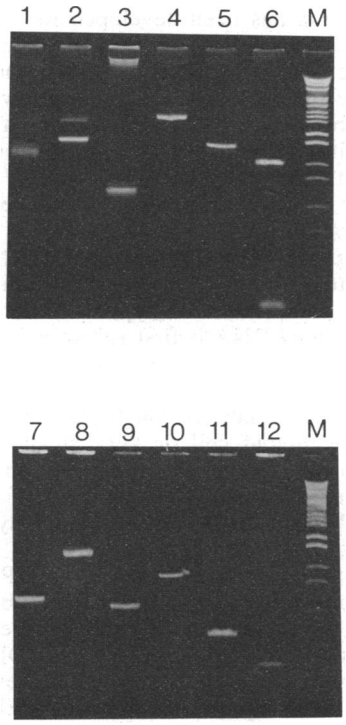

FIG. 2. Analysis of aliquots from a single-sperm (Table 2, sperm 12) PEP preparation for the presence of DNA sequences from 12 genetic loci. Each aliquot contained 1/24th of the PEP product. Lanes 1–12 show the PCR products obtained using primers specific for *D19S49* [132 base pairs (bp)], *D9S52* (152 bp), *APOC2* (101 bp), *PTH* (177 bp), X chromosome-linked *STS* (144 bp), pa (122 bp), *LDLR* (96 bp), *HBG2* (139 bp), *D3S2* (95 bp), *D3S3* (128 bp), *D3S11* (75 bp), and *D3S12* (62 bp), respectively. Lanes M show a *Msp* I digest of pBR322.

DISCUSSION

Methods for amplifying a random collection of cellular DNA sequences rather than one specific sequence have been used for cloning portions of microdissected chromosomes (20, 21) and in a scheme to select for genomic DNA segments that are capable of binding specific proteins (22). Amplification is achieved by ligating specific sequences to both ends of the fragments and by using primers complementary to these specific sequences for a PCR. In these approaches considerable manipulation of the sample is required before amplification including isolation of double-stranded DNA, restriction enzyme digestion, and ligation. From the data presented

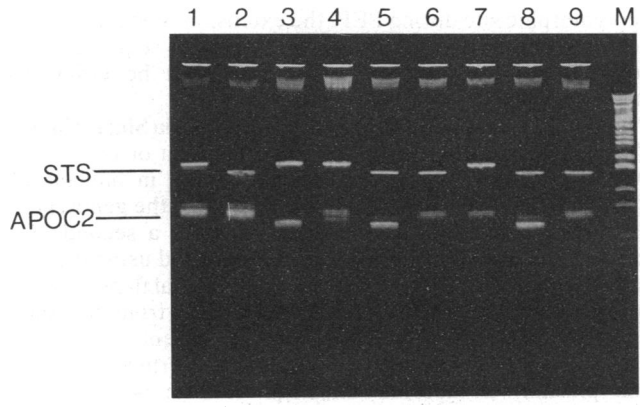

FIG. 3. *APOC2* and *STS* (or *STS* pseudogene) amplification products from nine sperm (lanes 1–9, respectively). Each lane contains a mixture of the products from the two amplifications, each using 1/24th of the PEP product. Lane M contains a *Msp* I digest of pBR322. Two bands can be seen for each sperm. The upper and lower *STS* bands represent the Y chromosome-linked (153 bp) and X chromosome-linked (144 bp) genes, respectively, and the two *APOC2* bands are the two alternative alleles in this individual.

in these studies, it is not possible to estimate what fraction of the total starting DNA was represented in the PCR product. We did not think it was likely that these approaches would be useful when starting with a single cell since it was expected that portions of the single genome would be lost during the many manipulations required.

On the contrary, PEP does not involve extensive manipulations of the sample. Only cell lysis, the addition of reagents, and repeated rounds of primer extension are required. Because of the random nature of the primers, it is also unlikely that any one primer-extension product will undergo primer extension at a subsequent cycle more than any other primer-extension product. Our data are consistent with these expectations. With our current protocol, we are 95% confident that PEP produces a minimum of 30 copies of not less than 78% of the DNA sequences present in a single cell.

Our first PEP experiments (data not shown) were carried out with random hexanucleotides and were not successful using different combinations of annealing (25–37°C) and extension (50–72°C) temperatures. Switching to random 15-mers dramatically improved our results. The PEP protocol we report uses 5 units of *Taq* polymerase and primer at 33 μM. Two units of *Taq* polymerase and primer at 20 μM also works (data not shown) but the results of these experiments have not been subjected to the extensive quantitative evaluation reported above. It is possible that further changes in primer length, primer-extension conditions, dNTP concentration, or other aspects of the PEP reaction could lead to increases in copy number beyond what has been achieved.

We do not know the size distribution of the PEP products since the amount of DNA present after 50 cycles is too low to be detected by conventional means. Some molecules must be greater than 335 bp in length, which is the size of the longest first-round PCR product (*HBG2*, ref. 12) in the data reported here. Recent experiments have shown that some PEP fragments must be at least 800 bp in length (data not shown) but their copy number has not been quantitated.

Could the use of a random-primer mixture introduce mutations into the PEP products and will this affect the subsequent analysis of specific sequences by PCR? This is not likely for the following reason. For a specific locus to be amplified from a PEP product in a subsequent experiment, the PEP product must contain at least one molecule with the specific upstream and downstream primer sequences that flank the target region for the particular locus under investigation. If random 15-mers anneal to the target region at the polymorphic site during PEP, the extension products will not contain the upstream-specific PCR primer sequences and exponential amplification by PCR cannot be efficiently achieved.

Implications of PEP for Single Cell Analysis in Single Gamete Typing. Single gamete typing using a sperm or oocyte has been used to study genetic recombination in humans (4, 12–15), mice (16), and cattle (23). However, the genotype of any one sperm could not be confirmed by a second PCR analysis. Not only can the typing be repeated using the PEP method but the possible exchange of additional flanking DNA polymorphisms detected in other aliquots from the same sperm could confirm rare recombination events.

The original sperm typing method for ordering DNA polymorphisms was laborious and required a series of independent three-point crosses (13, 24, 25). The development of the PEP procedure, however, will allow a more conventional multipoint mapping strategy where the same set of meiotic products can be studied for a number of polymorphisms. A very conservative estimate is that we can study at least 24 2.5-μl aliquots out of 60 μl of PEP product from a single sperm. The ability to type this many polymorphisms, coupled with the fact that informative males are easily found for virtually any combination of polymorphisms due to the very large number of sperm donors that are available, will allow map construction using standard multipoint mapping statistical procedures. In addition to determining order, the large number of sperm that can be examined would also allow the recombination fraction to be estimated with great accuracy. Sperm typing is also amenable to automation, which could enhance the speed of mapping. Aliquots of the semen samples would be used to determine which donors were informative for the polymorphisms to be mapped. Once multiply informative donors were identified, single sperm from those donors would be sorted, their genome would be amplified using PEP, and aliquots would be typed for the appropriate polymorphisms as described above.

The PEP approach to multipoint mapping by sperm typing would be possible in any species where single sperm can be isolated and would be especially valuable in those cases where selected breeding is difficult or extensive pedigrees are unavailable. In species where multiple oocytes can be obtained the same approach could be used (16, 26).

Preimplantation Genetic Diagnosis and Small DNA Samples. Another potential use of the PEP procedure is in preimplantation genetic disease diagnosis. A number of studies in mice and humans have shown that, after *in vitro* fertilization, analysis of DNA in individual cells from early embryos (6–9) or the polar body accompanying the oocyte before fertilization (10, 11) could be a useful tool for prenatal diagnosis before implantation. Since a PCR is not error free, clinical use of single-cell PCR data is accompanied by some risk since it has not been possible to confirm the genotype of any one embryonic cell or polar body. Prospective patients need to be counseled as to the risk of errors because, after implantation and a confirmatory chorion villus sampling or amniocentesis, a therapeutic abortion might still be warranted. Risk assessment based on a probabilistic method that takes PCR errors into consideration has been developed for both autosomal and sex-linked dominant and recessive diseases diagnosed using single-cell analysis (27, 28). However, these risks could be appreciably lowered if the PEP procedure was first carried out on the embryonic cell or polar body so that the genotype could be confirmed. In addition, the number of loci studied could be substantially increased.

Often, only very small DNA samples are available for forensic analysis or studies of ancient DNA. The PEP procedure could prove useful in these cases. When more molecules are examined in any one PCR experiment, the likelihood that the sample will be typed accurately increases. Also, if the isolated DNA is first subjected to PEP, significantly more genetic loci could be examined from an initially limited sample.

Considerations in Analyzing Single Diploid Cells and DNA. When a single diploid cell or very small cell-free DNA samples are to be typed, careful consideration must be given to the size of each aliquot and the number of aliquots taken from the PEP reaction. This is due not only to the small amounts of starting material but also to the relatively few copies made of each DNA segment during PEP (average, 60). In single diploid cell analysis, the sample subjected to PEP contains two copies of each autosomal gene. PEP should produce approximately equal numbers of DNA fragments representing both alleles at each locus. However, if the size of the aliquot taken from the sample after PEP is too small, the two different allelic DNA fragments may not be present in equal amounts due to sampling error. One allele might be missing altogether, although typing another aliquot could reveal the missing allele. Thus, the genotype determined by typing only one small aliquot may be different from that of the original sample. This is also true for very small cell-free forensics or ancient DNA samples that, in addition, could have had a biased representation of the two alleles even before PEP. Thus both aliquot size and number are important

for correct typing. Statistical considerations and suggested experimental approaches relevant to this sampling problem are discussed in Navidi *et al.* (29).

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