Two-dimensional DNA fingerprinting of human individuals

(DNA polymorphisms/minisatellites/variable number of tandem repeat sequences/denaturing gradient gel electrophoresis/gene mapping)

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Communicated by Leonard S. Lerman, November 28, 1988 (received for review June 9, 1988)

ABSTRACT The limiting factor in the presently available techniques for the detection of DNA sequence variation in the human genome is the low resolution of Southern blot analysis. To increase the analytical power of this technique, we applied size fractionation of genomic DNA restriction fragments in conjunction with their sequence-dependent separation in denaturing gradient gels; the two-dimensional separation patterns obtained were subsequently transferred to nylon membranes. Hybridization analysis using minisatellite core sequences as probes resulted in two-dimensional genomic DNA fingerprints with a resolution of up to 625 separated spots per probe per human individual; by conventional Southern blot analysis, only 20–30 bands can be resolved. Using the two-dimensional DNA fingerprinting technique, we demonstrate in a small human pedigree the simultaneous transmission of 37 polymorphic fragments (out of 365 spots) for probe 33.15 and 105 polymorphic fragments (out of 625 spots) for probe 33.6. In addition, a mutation was detected in this pedigree by probe 33.6. We anticipate that this method will be of great use in studies aimed at (i) measuring human mutation frequencies, (ii) associating genetic variation with disease, (iii) analyzing genomic instability in relation to cancer and aging, and (iv) linkage analysis and mapping of disease genes.

The possibility to identify DNA sequence heterogeneity is of major importance for the analysis of genetic diseases and genomic instabilities. This identification depends on the availability of probes that detect variable sites in the genome and on the resolution of electrophoretic separation techniques for the analysis of DNA restriction fragments.

The discovery of hyperpolymorphic VNTR (variable number of tandem repeat) DNA sequences or minisatellites has greatly facilitated studies of genetic variation in the human population (1–6). It has been demonstrated that so-called core probes derived from minisatellites can be used to simultaneously detect a large number of hyperpolymorphic VNTR loci, dispersed in the genome, to provide genetic fingerprints of human individuals (7, 8). Core sequences have been successfully applied in the analysis of tumors for genetic instability (9) and in linkage analysis of genetic diseases (3). Such applications rely on the resolution of Southern blot hybridization analysis, which is based on the gel electrophoretic separation of genomic DNA restriction fragments according to size (10). One-dimensional separation of DNA fragments allows only ≈30 hypervariable minisatellite fragments to be resolved (ref. 3; this publication).

It has been demonstrated that by combining ordinary size separation with sequence separation in denaturing gradient gels, all restriction fragments in an EcoRI digest of the Escherichia coli genome can be resolved (11-13). To investigate whether this principle can be applied to the analysis of DNA sequence variation in the mammalian genome, we separated genomic DNA restriction fragments of human individuals according to size and base-pair composition by neutral and denaturing gradient polyacrylamide gel electrophoresis, respectively. By subsequent transfer of the electropherograms to nylon membranes and hybridization with radiolabeled minisatellite core sequences as probes, high-resolution DNA fingerprints were obtained. We show that many polymorphic DNA restriction fragments can be detected in the molecular size region of fragments <3 kilobase pairs (kbp), which is not accessible for conventional Southern hybridization analysis. We demonstrate the applicability of this technique in genetic studies on humans by showing that a large number of transmitted polymorphic spots can be simultaneously followed in a two-generation human pedigree of three members.

MATERIALS AND METHODS

DNA Isolation and Restriction Enzyme Digestion. Genomic DNAs, isolated from peripheral blood lymphocytes according to standard procedures, were a kind gift from E. Bakker. DNAs were digested with the restriction endonucleases Hae III or HindI (BRL) under conditions recommended by the manufacturer.

Electrophoretic Separations. Agarose gel electrophoresis of 5 µg of DNA restriction fragments was performed in a horizontal 1.2% gel in 1× TAE (40 mM Tris-HCl, pH 7.4/20 mM sodium acetate/1 mM NaEDTA) at 65 V for 14–30 hr. Gels were stained for 10 min in a solution containing ethidium bromide (EtBr) at 0.1 µg/ml followed by destaining for at least 30 min. Two-dimensional separations of 5 µg of DNA restriction fragments were performed in 1-mm-thick polyacrylamide gels (acylamide/bisacrylamide, 37:1) using a gel apparatus that was essentially the same as the one described by Fischer and Lerman (12). The first dimension was run in a neutral 6% gel at 50°C for 2 hr at 250 V in 1× TAE. The separation patterns were visualized by staining the gel in the dark with EtBr (0.1 µg/ml) for 10 min, followed by destaining for at least 30 min. The 0.34- to 2.8-kbp region (probe 33.15) or the 0.54- to 10-kbp region (probe 33.6) of the lane was quickly cut out of the gel and applied to a 6% polyacrylamide gel containing a 10–75% linear concentration gradient of denaturant (100% denaturant = 7.0 M urea/40% formamide) parallel to the direction of electrophoresis. This gradient was found to give optimal separation patterns for the VNTR sequences. Gels were poured by mixing two solutions, containing the desired boundary denaturant concentrations, in a linear gradient maker with a peristaltic pump. After electrophoresis for 12 hr at 225 V and 60°C, the gel was stained with EtBr as described above.

Transfer of Separation Patterns. DNA separation patterns in agarose gels were capillary transferred to a nylon membrane (Nytran 13N, Schleicher & Schuell; Zetaprobe, BioRad) in 0.4 M NaOH/0.6 M NaCl for 12 hr. After transfer, the filter was rinsed in 2× SSC (1× SSC = 150 mM NaCl/15 mM NaEDTA).

Abbreviations: EtBr, ethidium bromide; VNTR, variable number of tandem repeats.
sodium citrate), air dried, baked for 1 hr at 80°C, and irradiated with 302-nm UV light (Transilluminator, UVP Products, San Gabriel) for 45 s, which was found to be optimal for cross-linking DNA to the filter (results not shown). DNA separation patterns in denaturing gradient polyacrylamide gels were fragmented by irradiating the gel with 302-nm UV light (Transilluminator, UVP Products) for 4 min, which was found to be optimal (results not shown). Before transfer, the gel was boiled for 5 min in 1× TBE (89 mM Tris-borate, pH 8.0/89 mM boric acid/2 mM NaEDTA) and subsequently placed in room solution at room temperature. Transfer to a nylon membrane (Nytran 13N, Schleicher & Schuell; Zetabind, Bio-Rad) was achieved by semidy electroblotting at 400 mA (6–28 V) between horizontal graphite plates. Electrophoresis was performed twice for 45 min between 10 Whatman 3MM paper sheets, which were soaked in fresh 1× TBE between the two transfers. After transfer, the filter was rinsed in 2× SSC, air-dried, baked for 1 hr at 80°C and irradiated for 45 s with 302-nm UV light to cross-link the DNA fragments to the filter.

**Probe Preparation and Labeling.** The probe was prepared by using T4 kinase (Boehringer Mannheim) to individually phosphorylate the 5'-hydroxyl groups of two partially complementary and overlapping oligonucleotides, each representing the complete 33.15 (5'-AGAGGTGCGAGTGG-3' and 5'-CCACCTCCACCTGC-3') or 33.6 (5'-AGGGCTGGAGG-3' and 5'-AGGGCTGAGG-3') minisatellite core sequence (7). Subsequently, the two 33.15 or 33.6 oligonucleotides were mixed and allowed to anneal at 57°C for 1 hr, followed by ligation according to standard procedures (14). The synthetic probes thus prepared had an average length of 500 bp or more. Occasionally, T4 kinase phosphorylation and ligation were repeated to increase the average length of the probe. The ligation products (20 ng) were [α-32P]dCTP-labeled either by the random-primer oligolabeling method (Boehringer Mannheim) or by self-priming, after boiling for 5 min and reannealing at 37°C in the presence of 1 unit of Klenow enzyme (Boehringer Mannheim)/2 μM dNTP/50 mM Tris-HCl, pH 7.2/10 mM MgCl₂. Specific activities of 3 × 10⁹–8 × 10⁹ cpm/μg were obtained.

**Hybridization Analysis.** Filters were prehybridized in 5× SSC/20 mM sodium phosphate, pH 7.2/1% SDS/1 mM NaEDTA/heparin (50 μg/ml) for 2 hr at 65°C. After adding denatured probe at a concentration of 1 × 10⁶ cpm/ml, hybridization was performed for 12 hr at 65°C. The filter was washed three times for 5 min at room temperature and three times for 20 min at 65°C in 2.5× SSC/0.1% SDS. Autoradiography was performed for 12–48 hr at −80°C using fine intensifying screens and XAR-5 film (Kodak). For subsequent rehybridizations of the filters, the probe was removed by boiling the filter for 20 min in a solution containing 0.01× SSC and 0.1% SDS. Filters were rinsed in 2.5× SSC and hybridization analysis was performed as described above. Two-dimensional spot patterns were interpreted by eye examination, using grids to quantitate the spots and to match individual two-dimensional fingerprints.

**RESULTS**

In Fig. 1, Southern blot autoradiographs are shown of a human pedigree (of six members) obtained after prolonged electrophoresis in agarose gels and by using the synthetic probes derived from minisatellite core sequences 33.15 and 33.6 (7) on Hae III- and HinfI-digested genomic DNA.

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**Fig. 1.** Southern blot hybridization analysis of DNAs isolated from six members of a human pedigree (DL63; schematically depicted above the lanes in the autoradiographs) and one from an unrelated individual (DL 63.5), digested with Hae III or HinfI, and using 35.15 (Left) or 33.6 (Right) minisatellite core sequences as probe. The autoradiograph on the Right was obtained by rehybridizing the filter that was used to obtain the autoradiograph on the Left.
number of simultaneously transmitted polymorphic bands were detected in this pedigree in the high molecular weight area of restriction fragments. For the restriction enzyme HinII, this number was 28 for probe 33.15 and 33 for probe 33.6. With Hae III, the number was 28 for probe 33.15 and 22 for probe 33.6. After Hae III digestion, the size range of bands detected by probe 33.6 was found to be smaller than after digestion with HinII, which could be an indication for the presence of Hae III recognition sites in the minisatellites homologous to this probe.

When genomic DNA was digested with Hae III and subjected to a two-dimensional separation (on the basis of size and base-pair sequence in neutral and denaturing gradient polyacrylamide gels, respectively), clusters of restriction fragments were observed in the EtdBr-stained two-dimensional gel as shown in Fig. 2 (Left). A large cluster is seen in the upper part of the gel, containing low concentrations of denaturant, and a smaller cluster is located near the bottom of the gel, which contains the highest concentration of denaturant.

Hybridization analysis of VNTR sequences detected with probe 33.6 resulted in a more or less evenly spread spot pattern. The total number of spots observed for probe 33.6 under the stringency of washing applied here (2.5X SSC) was 545 for this individual (DL 63.4 from the pedigree shown in Fig. 1). Slightly smaller numbers were obtained when the stringency was increased to 1X SSC (results not shown).

We subsequently analyzed three members of the pedigree described above (mother, father, and a son) by two-dimensional DNA fingerprinting. For optimal comparisons a 30-cm-wide version of the gel apparatus originally described by Fischer and Lerman (13) was constructed and used in these experiments so that up to six individuals could be compared on one denaturing gradient gel. Close inspection and comparison of individual spot patterns of the two parents obtained with probes 33.6 (Fig. 3) revealed a total number of 569 spots for the father, 607 for the mother, and 625 for the son. Between the two parents, 150 spot polymorphisms were observed, 105 (70%) of which were transmitted to the son (52 of maternal and 53 of paternal origin). Details of the separation patterns are shown in Fig. 3 (Lower Left). Using probe 33.6, we detected a fragment in the son that was not present in the mother or the father (Fig. 3 Lower Right) and four fragments, common to the parents, could not be detected in the son (two examples are shown in Fig. 3 Lower Right). With probe 33.15, considerably less VNTR-containing fragments were detected than with probe 33.6 (Fig. 4). Among the 372, 290, and 365 spots for the father, mother, and son, respectively, 50 spot polymorphisms could be detected. Among the 37 transmitted spot polymorphisms (74%), 17 were of paternal and 20 were of maternal origin. Some of these spot polymorphisms are shown in detail in Fig. 4 (Lower).

**DISCUSSION**

With the two-dimensional DNA fingerprinting system presented here, we were able to distinguish up to 625 spots per individual for probe 33.6 and 372 for probe 33.15. Since we did not observe severe clustering of spots, it is likely that we have resolved all VNTR sequences belonging to the sets of sequences detected with these probes. The difference between the two probes in number of spots detected could therefore be due to a different copy number of these sets of repetitive sequences. In addition to 33.15 and 33.6, we have also used other core sequences (5) as probes in two-dimensional DNA fingerprinting and obtained comparable results. In this respect, VNTR sequences appear to exhibit an exceptional distribution of spots over a considerable part of the denaturing gradient in the second-dimension gel. By contrast, as illustrated in Fig. 2 (Left), total genomic DNA digests have a tendency to cluster in the second dimension. Most spots were found in the 20–50% denaturant range and should therefore have a high to medium AT/GC ratio of their lowest melting domain. Although the number of repeat units of any VNTR locus is high enough to generate a melting domain, both 33.15 and 33.6 are G+C-rich. Therefore, the gradient level of the majority of homologous restriction fragments in the two-dimensional pattern cannot be determined by the VNTR sequences themselves. Instead, the position of most of the spots in the gradient will be determined by sequences adjacent to the VNTR regions. Virtually all VNTR alleles with a particular locus are therefore iso-

![Fig. 2. Two-dimensional DNA fingerprint analysis of a human individual (DL 63.4). The two-dimensional separation pattern is shown after EtdBr staining (Left) and after hybridization analysis of the nylon replica filter with probe 33.6 (Right).](image-url)
This characteristic and the fact that in the two-dimensional system virtually no sequences detected with a particular core probe are lost in a smear allows one to identify them.

When individuals from a human pedigree were analyzed by two-dimensional DNA fingerprinting, the majority of spots detected with both probes were found to be monomorphic (75–85%). However, a large number of spot polymorphisms (up to 150 for probe 33.6) were observed. Evidence for heterozygosity of the parents at particular VNTR loci detected by probe 33.6 was provided in four cases in which the parents shared a spot that could not be demonstrated in the son (Fig. 3 Lower Right). The percentage of spot polymorphism transmitted to the son was found to be 70% for both probes 33.6 and 33.15. This phenomenon could be due to clustering in the genome of VNTR loci or to the presence of one or more Hae III restriction sites in the minisatellites themselves. In the latter case, several polymorphic spots could stem from the same minisatellite locus, thereby resulting in a number of spots being cotransmitted as minisatellite haplotypes (3). This possibility is supported by the data in Fig. 1 (Right), which indicate the presence of Hae III sites in large alleles detected by probe 33.6 in these individuals. The spot present in the son but not in the parents is likely to have arisen by unequal exchange between two VNTR regions rather than by point mutation in a VNTR region (6).

On the basis of the results obtained with VNTR core sequence probes, we anticipate that two-dimensional DNA fingerprinting can be applied in a number of different areas in genetic research. The method should be useful in the analysis of large parts of the genome for measuring mutation frequencies (15) and for detecting putative changes in VNTR loci or other unstable DNA regions during tumor induction and growth (9, 16) or during aging (17).

Two-dimensional DNA fingerprinting can be used in association studies and to extend and improve linkage analysis and gene mapping. In this respect, it should be noted that the introduction of the denaturing gradient separation principle offers a solution to the problem of not being able to identify the same VNTR locus in different pedigrees, which effectively constrains widespread application of one-dimensional
DNA fingerprinting to genetic analysis. Sequence-specific separation allows one to identify alleles with a particular locus in different pedigrees on the basis of their position on the same isotherm (see above). Furthermore, by using locus-specific probes in parallel experiments, each VNTR core homologous spot in a two-dimensional gel can be identified by comparison.

We especially acknowledge Dr. Stuart Fischer's expert advice and kind hospitality during the initial stages of this work when one of us (A.G.U.) stayed in his laboratory. We thank Dr. Leonard S. Lerman for many helpful comments on the manuscript and Drs. J. A. Gossen, J. H. J. Hoeijmakers, H. Schellekens, and G. J. J. M. Trommelen for helpful discussions. We also thank Dr. E. Bakker (Department of Human Genetics, State University of Leiden, Leiden, The Netherlands) for his kind gift of DNAs from human pedigree DL63 and Mr. E. J. van de Reyden for the preparation of the photographs. Part of this research was supported by Senetek PLC. This work is the subject of a patent application.