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Analysis of the VNTR Locus DIS80 by the PCR Followed by High-Resolution PAGE

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Summary

Allelic data for the D1S80 locus was obtained by using the PCR and subsequent analysis with a high-resolution, horizontal PAGE technique and silver staining. Compared with RFLP analysis of VNTR loci by Southern blotting, the approach described in this paper offers certain advantages: (1) discrete allele resolution, (2) minimal measurement error, (3) correct genotyping of single-band VNTR patterns, (4) a nonisotopic assay, (5) a permanent record of the electrophoretic separation, and (6) reduced assay time. In a sample of 99 unrelated Caucasians, the D1S80 locus demonstrated a heterozygosity of 80.8% with 37 phenotypes and 16 alleles. The distribution of genotypes is in agreement with expected values according to the Hardy-Weinberg equilibrium. Furthermore, the observed number of alleles and the level of heterozygosity, obtained through the protocol described here, were congruent with each other in accordance with the expectation of a mutation-drift equilibrium model for a single, homogeneous, random-mating population. Therefore, the analysis of D1S80 and similar VNTR loci by amplified fragment length polymorphism (AMP-FLP) may prove useful as models for population genetic issues for VNTR loci analyzed by RFLP typing via Southern blotting.

Introduction

Identity tests, as performed in the fields of paternity and forensics, rely on the detection of genetic differences among individuals. At present, highly polymorphic loci whose alleles are the result of VNTRs are the most informative genetic markers for genetic characterization. Although extremely effective for VNTR analyses, the RFLP methodology via Southern blotting (Southern 1975) is time consuming and requires an isotopic assay to achieve the sensitivity necessary to detect VNTR alleles in samples containing as little as 10–50 ng of human DNA samples (Budowle and Baechtel 1990). Additionally, because of the inability of the RFLP technology to resolve discretely the alleles of most VNTR loci, statistical analyses that are different from those used for traditional genetic marker systems have been required (Budowle et al., in press).

The PCR (Saiki et al. 1985) offers a viable alternative to RFLP analysis of VNTR loci, particularly in situations where limited quantities of DNA are available. The use of the PCR can obviate the need for isotopic detection and reduce assay time and cost. With appropriate VNTR loci and high-resolution discontinuous buffer electrophoretic systems in polyacrylamide gels (Allen et al. 1989; Budowle and Allen 1990), amplification of specific DNA sequences by the PCR could prove useful for identity testing, population genetics, and disease susceptibility studies. In fact, the D17S30 (also designated D17S5) locus (Horn et al. 1989) and the 3′ hypervariable region of the apolipoprotein B gene (Boerwinkle et al. 1989; Ludwig et al. 1989) have been

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analyzed using the PCR and subsequent electrophoretic separation of the amplified fragments.

This paper describes the results of the analysis of PCR-amplified products of the VNTR locus D1S80 (Nakamura et al. 1988). The procedure resolves alleles of D1S80 into discrete entities, uses an inexpensive silver stain for detection, and provides a permanent record of the electrophoretic separation. With an analytical system that enables resolution of discrete alleles and therefore permits correct genotyping of VNTR profiles, it will now be possible to apply the conventional formula of the Hardy-Weinberg rule (i.e., χ^2 analysis on observed and expected genotype classes). This will allow for an evaluation of the goodness of fit of the genotype distributions of the particular VNTR locus for a sample population. Moreover, with the discrete resolution of alleles it will be possible to evaluate the appropriateness of classical population genetic models of allele frequency distributions at this locus to validate an assumption of genetic homogeneity of the population from which the sample is derived.

Material and Methods

Whole blood was obtained in EDTA Vacutainer tubes by venipuncture from 100 unrelated Caucasian donors at the FBI Academy. The DNA was extracted as described previously (Budowle and Baechtel 1990). Purified DNA from a two-generation (10 individuals) and a four-generation family (18 individuals) was provided by M. Skolnick (University of Utah, Salt Lake City, UT).

Amplification of D1S80 was achieved using the primers described by Kasai et al. (in press). The primers were 5′-GAAACTGGCCTCCAAACACTGCCCGCCG-3′ and 5′-GTCTTGTTGGAGATGCACGTGCCCCTTGC-3′. Each sample that was amplified contained 100 ng DNA, 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelation, 2.5 units of Amplitaq™ DNA polymerase (Perkin Elmer–Cetus), 1 μM of each primer, and 200 μM of each dNTP. The total volume of each sample was 50 μl. Each sample was overlaid with 50 μl of mineral oil. The PCR was carried out in a Perkin-Elmer Thermocycler for 25 cycles. Each cycle consisted of 1 min at 95°C for denaturation, 1 min at 65°C for primer annealing, and 8 min at 70°C for primer extension. After amplification, the mineral oil was removed and the samples were stored at either 4°C or −20°C prior to electrophoretic analysis.

Ultrathin-layer polyacrylamide gels (5% T, 3% C; 400 μm thick) were cast onto Gelbond (FMC, Rockland, ME) using the flap technique (Allen 1980). The gels were cross-linked with piperazine diacrylamide (Hochstrasser et al. 1988) (Bio-Rad Laboratories, Richmond, CA). All gels contained 7.1% glycerol and 33 mM Tris-sulfate buffer, pH 9.0. If rehydratable polyacrylamide gels were used (Allen et al. 1989; Budowle and Allen 1990), they were rehydrated in a solution containing 33 mM Tris-sulfate, pH 9.0, and 7.1% glycerol. The trailing ion, contained in 2% (wt/vol) agarose plugs, was 0.14 M Tris-borate, pH 9.0. Bromophenol blue (a final concentration of 0.01%) was added to the electrode buffer to serve as a dye marker for the discontinuous buffer boundary. The electrophoretic setup was similar to that described by Allen et al. (1989) and Budowle and Allen (1990). The distance between the edges of the agarose plugs was 10 cm. The amplified fragment length polymorphisms (AMP-FLPs) of D1S80 were absorbed into fiberglass applicator tabs (2.5 × 5.0 mm, Pharmacia-LKB, catalog no. 1850-901), lightly blotted, and applied to the gel surface 1 cm from the cathode. The conditions for electrophoretic separation were similar to those described previously for rehydratable polyacrylamide gels (Allen et al. 1989). Electrophoretic separation was stopped when the bromophenol blue dye front reached the anodal wick. Following electrophoresis, the gels were stained with silver, according to the conditions described in table 1, so the pattern could be visualized directly.

Hybridization analysis of the PCR-amplified products (or AMP-FLPs) subsequent to electrophoresis in the ultrathin-layer polyacrylamide gels was accomplished using a passive blotting procedure. After electrophoresis, the DNA in the gel was denatured by washing the gel in 0.4 M NaOH for 5 min. A nylon membrane (Zeta Probe, Bio-Rad Laboratories, Richmond, CA), prewetted in 0.4 M NaOH, was placed directly on the gel surface, and, subsequently, a blot pad (BRL, Gaithersburg, MD) was placed on the membrane. Transfer time was 1 h at ambient temperature. After transfer, the membrane was washed briefly in a solution containing 2 × SSC (20 × SSC = 1,753 g NaCl and 88.2 g sodium citrate/l, pH 7.0) (Maniatis et al. 1982) and 0.2 M Tris, pH 7.5. The membrane was blotted between two Whatmann 1 MM papers and baked in an oven at 80°C for 30 min. The membrane was wrapped in plastic wrap and stored at −20°C. The probe pMCT118 (for locus D1S80) was provided by Y. Nakamura and R. White (Howard Hughes Medical Institute, Salt Lake City, UT). Random primer labeling was accomplished according to the manufacturer's in-

Table 1

Protocol for Silver-Staining AMP-FLP Gels

Step	Time
Place gel in 10% ethanol solution	5 min
Oxidize gel in 1% nitric acid solution	3 min
Rinse gel in distilled water	A few seconds
Place gel in 0.012 M silver nitrate solution	20 min
Decant silver nitrate and rinse gel in distilled water	A few seconds
Reduce gel in a solution containing 0.28 M sodium carbonate (anhydrous) and 0.019% formalin; several changes of reducing solution may be necessary; the solution should be changed when it turns brown	Will depend on desired intensity of image; image develops before eye
Stop reduction process with 10% glacial acetic acid	2 min
Place gel in distilled water	2 min
Air dry gel for permanent record	. . .

structions contained within the BRL Random Primer DNA labeling system kit or according to the method of Feinberg and Vogelstein (1983, 1984). Hybridization and stringency washes were carried out according to the method of Budowle and Baechtel (1989). The labeled DNA duplex was detected by autoradiography using Kodak XAR film and Dupont Cronex Lightning Plus intensifying screens.

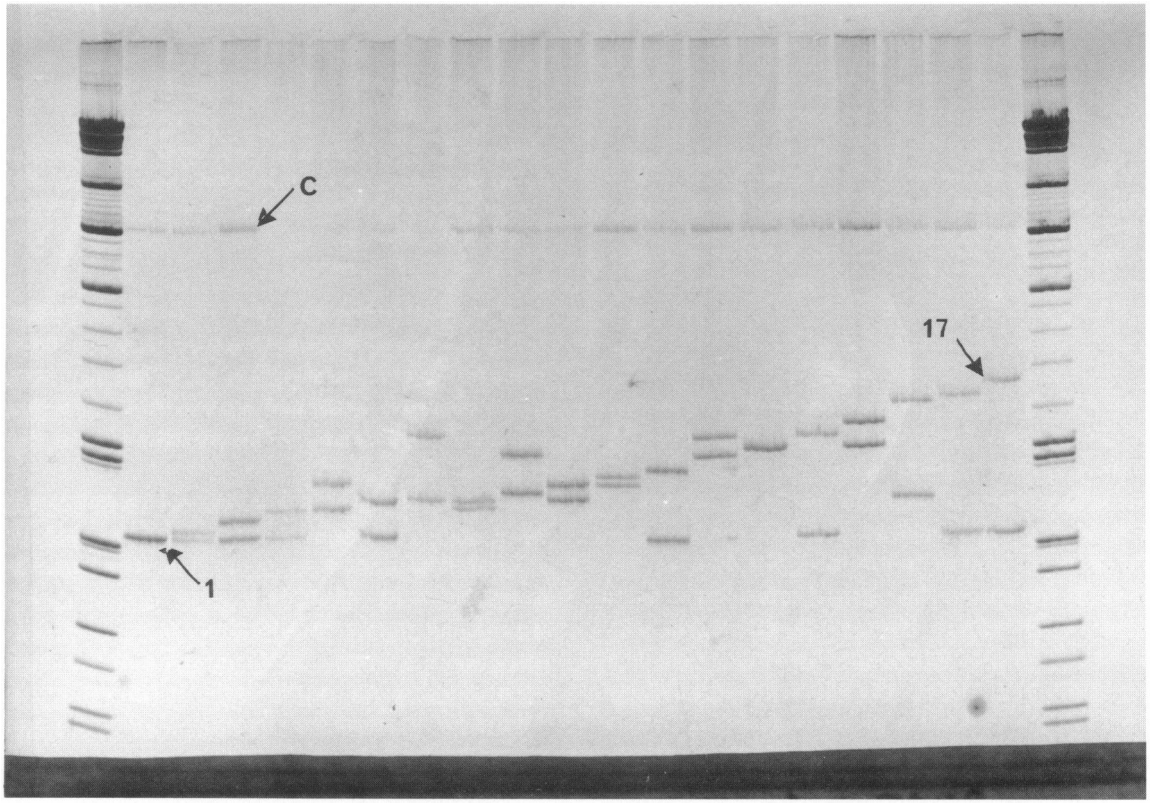

Figure 1 Silver-stained AMP-FLP gel displaying D1S80 profiles. The D1S80 types from left to right are 1-1, 2-1, 3-1, 4-1, 7-4, 5-1, 12-5, 5-4, 10-6, 7-5, 8-7, 9-1, 12-10, 11-11, 12-1, 13-11, 15-5, 16-1, and 17-1. C = constant band. The size standards are a combination of the 1-kb and 123-bp ladder (BRL, Gaithersburg, MD). The cathode is at the top.

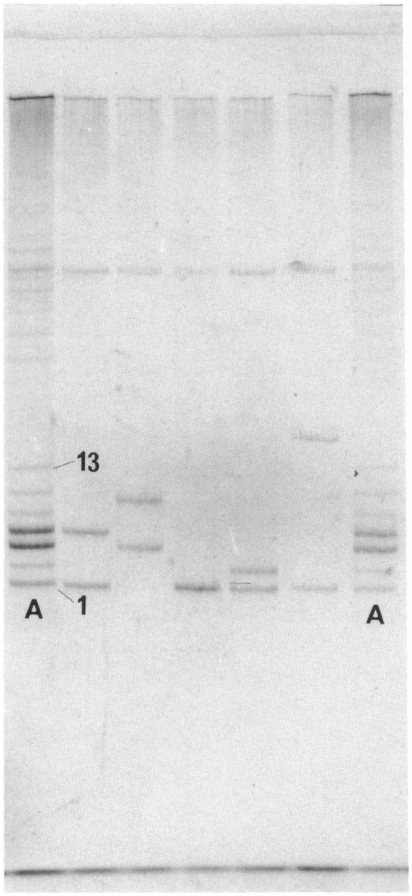

Figure 2 Silver-stained AMP-FLP gel displaying a ladder of composite alleles and D1S80 profiles. The ladder (A) is composed of alleles 1, 3, 5, 7, 9, 11 and 13. The D1S80 types from left to right are 7-1, 10-5, 1-1, 3-1, and 15-1. The cathode is at the top.

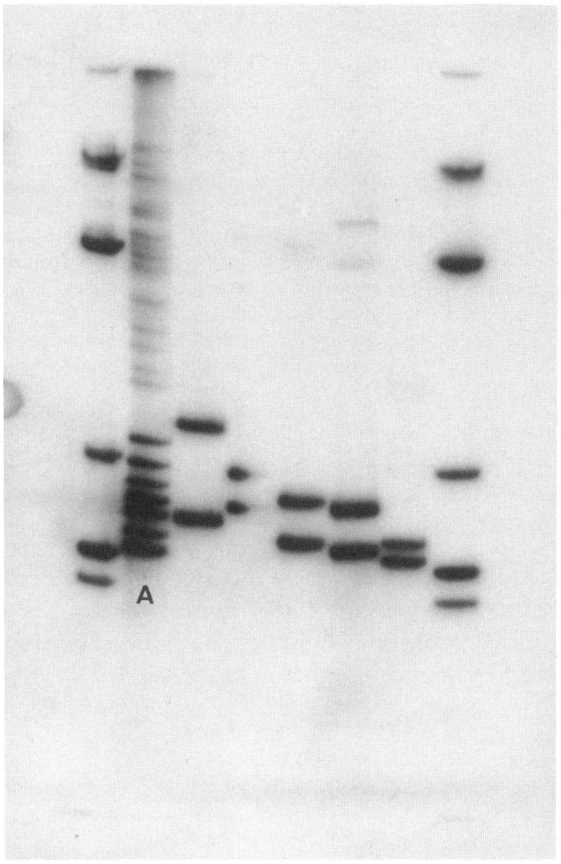

Figure 3 Autoradiogram of an AMP-FLP gel displaying the ladder of composite alleles (A) and D1S80 types. The pMCT118 probe was provided by Ray White and his colleagues (Howard Hughes Medical Institute, Salt Lake City, UT).

Results

Figure 1 shows that AMP-FLP analysis of D1S80 can be performed using the techniques described in this paper. Sixteen different alleles were observed in 99 unrelated Caucasians (one sample did not amplify by PCR). Each allele was completely resolved based on increments of the repeat unit of the VNTR locus. The length of the repeat unit has been determined to be 16 base pairs (Y. Nakamura, Howard Hughes Medical Institute, and T. Holm, GenMark, Salt Lake City, UT, personal communications). The alleles have been designated 1–17 (allele 14 has not yet been observed), where allele 1 is the smallest in length and allele 17 is the largest in length. (It should be noted that the allele designations are temporary and will eventually be based on the number of repeats.)

With this system the alleles can be designated specifically without determining base-pair size. Unknown samples can be compared with a "ladder" consisting of a composite of D1S80 alleles; thus, allele designations are much easier and measurement error is greatly reduced (fig. 2).

It should be noted that a constant (or monomorphic) band appears in each amplified sample (indicated by the arrow at point C in fig. 1). The band does not hybridize with the pMCT118 probe (fig. 3) and thus is a sequence unrelated to D1S80. However, shifts in the position of the constant band can indicate unaligned positioning of the sample tabs at the sample origin. Therefore, the constant band can serve an important function as an internal marker to minimize errors in AMP-FLP typing of D1S80.

The distributions of observable phenotypes and allelic frequencies for D1S80 in a Caucasian population

Table 2

Distribution of D1S80 Genotypes from 99 Unrelated Caucasians

Genotype	Number Observed
1-1	7
2-1	1
3-1	3
4-1	2
5-1	1
6-1	2
7-1	24
8-1	1
9-1	3
10-1	1
11-1	2
12-1	2
16-1	1
17-1	1
7-2	1
8-3	1
5-4	1
7-4	1
10-4	1
7-5	2
10-5	1
11-5	2
12-5	1
15-5	1
10-6	2
7-7	10
8-7	3
10-7	3
11-7	4
12-7	5
13-7	1
15-7	1
10-8	1
12-8	1
12-10	2
11-11	2
13-11	1

sample of 99 unrelated individuals are shown in tables 2 and 3, respectively. The observed heterozygosity is 80.8%. The distribution of the phenotypes is in Hardy-Weinberg equilibrium ($\chi^2 = 2.50$; df = 1; $.100 < P < .250$; Hardy-Weinberg formulation was calculated by comparing observed and expected genotypes; all classes with less than four events were pooled). Further, although not extremely informative (because of limited variation among family members), the two families demonstrated Mendelian inheritance of the AMP-FLP alleles (data not shown).

While hybridization assays do not appear to be necessary for routinely typing D1S80 AMP-FLP profiles, it may be desirable, at times, to use a probe to confirm that the AMP-FLPs truly represent the described locus or to increase the level of sensitivity of detection provided by silver staining. Figure 3 shows that the blotting approach can be used. As expected, after the PCR there should be more than adequate quantities of DNA for hybridization analysis. Although the patterns are weak, there can still be enough residual DNA left in the polyacrylamide gel for detection by silver staining (fig. 4).

Discussion

AMP-FLP analysis of the D1S80 locus offers advantages over the typing of other highly polymorphic VNTR loci by Southern blotting. With routine Southern blotting, the resolution of alleles that differ by one to a few repeat units may not possible. Therefore, the alleles from a sample population form a quasi-continuous distribution of allele sizes (Budowle et al., in press). However, the alleles associated with the D1S80 locus are resolved into discrete entities using the AMP-FLP analytical technique. This greatly reduces the chance of measurement error. In fact, typing of D1S80 AMP-FLP profiles now is similar to that used for conventional protein genetic marker systems. The efficiency of amplification or yield of PCR products is related to the length of the target site between the primers. For example, at the D17S30 (also designated D17S5) locus it was observed by Horn et al. (1989) that larger alleles could be amplified to a significantly less extent than smaller ones. However, all AMP-FLP D1S80 alleles examined to date are less than 700 bp in length. There is no apparent difference in band intensity between the largest (number 17) and the smallest (number 1) alleles (fig. 1). Thus, AMP-FLP analysis of D1S80 permits correct genotyping, not just phenotyping, of VNTR profiles. This is in contrast to the situation in RFLP analysis via Southern blotting, where correct genotyping may not always be possible. Larger DNA fragments, which contain more repeat sequences, are more readily detectable by hybridization assays than smaller fragments. Thus, some small-sized VNTR alleles may go undetected by RFLP analysis. Also, small-sized alleles can migrate off the end of the gel and therefore be undetectable (whereas, in AMP-FLP analysis, because of the versatility of the AMP-FLP gels, small-sized alleles need not migrate off the gel). Thus, single-band patterns, detected by RFLP analysis via Southern blotting, may or may not be true homozygotes (Budowle et al., in press).

Table 3

D1S80 Allele Frequencies from 99 Unrelated Caucasians

Allele	95% Lower Confidence Limit[a]	Point Estimate Frequency	95% Upper Confidence Limit[a]
1	.212	.293	.389
2	.002	.010	.055
3	.006	.020	.106
4	.008	.025	.078
5	.019	.045	.071
6	.006	.020	.071
7	.244	.328	.426
8	.013	.035	.093
9	.003	.015	.063
10	.025	.056	.120
11	.031	.066	.133
12	.025	.056	.120
13	.002	.010	.055
14	.000	.000	.047[b]
15	.002	.010	.055
16	.001	.005	.047
17	.001	.005	.047

[a] Confidence limits were calculated according to Goodman (1965).

[b] Value cannot be determined; therefore, an upper confidence limit of one observation (or .047 frequency) was used.

Since alleles are resolved discretely, there is little or no measurement error, and correct genotyping is permitted, the conventional formula of the Hardy-Weinberg rule can be applied to assess the goodness of fit of the distribution of genotypes for D1S80 ($\chi^2 = 2.50$, df = 1; $.100 < P < .250$; the Hardy-Weinberg formulation was calculated by comparing observed and expected genotypes [table 2]; all classes with less than four events were pooled). Therefore, it can be stated that the alleles associate randomly with each other at this locus and there is no detectable population heterogeneity. Additional evidence that the alleles associate randomly can be obtained from the allele frequency distributions. For example, using the allele frequency distribution shown in table 3, the expected number of distinct homozygote and heterozygote genotypes (and standard errors) under the Hardy-Weinberg model (see Chakraborty et al. [1988] for the theory) were computed. In the sample of 99 individuals, three homozygote genotypes and 34 different heterozygote genotypes (table 2) were observed, and the observed values are in close agreement with the expected values (3.37 ± 1.03 and 30.84 ± 3.56, respectively). In addition, using the theory described by Chakraborty et al. (1988) and Chakraborty (1990) the expected heterozygosity ($79.7\% \pm 8.9\%$) was obtained for the given observed number of alleles (16) in the sample. The expected value is almost identical to the observed heterozygosity (80.8%) in the sample. Alternatively, if the heterozygosity were fixed at the observed level, 13.3 ± 3.1 alleles would be expected for the 198 chromosomes sampled at this locus; this in turn shows that the observed number of alleles (16) is in close agreement with this expected value ($P = .236$, using the theory of Chakraborty [1990]). Since the classical Hardy-Weinberg test is known to lack adequate statistical power for detecting population heterogeneity, particularly when the number of alleles is as large as found in the context of VNTR studies (Ward and Singh 1970; Emigh 1980), these additional data provide further confidence that the VNTR polymorphism at the D1S80 locus in a Caucasian population satisfies the basic population genetic premises to make it useful for forensic applications. AMP-FLP analysis of D1S80 and similar VNTR loci may, therefore, be useful as models for population genetics issues for the more complex VNTR profiles detected by RFLP analysis via Southern blotting.

In conclusion, a simple, discontinuous, horizontal PAGE approach followed by silver-staining techniques was used to type AMP-FLPs from the D1S80 locus. Silver staining of AMP-FLP profiles offers an inexpensive, nonmutagenic assay that provides a permanent record of the actual electrophoretic separation (unlike ethidium

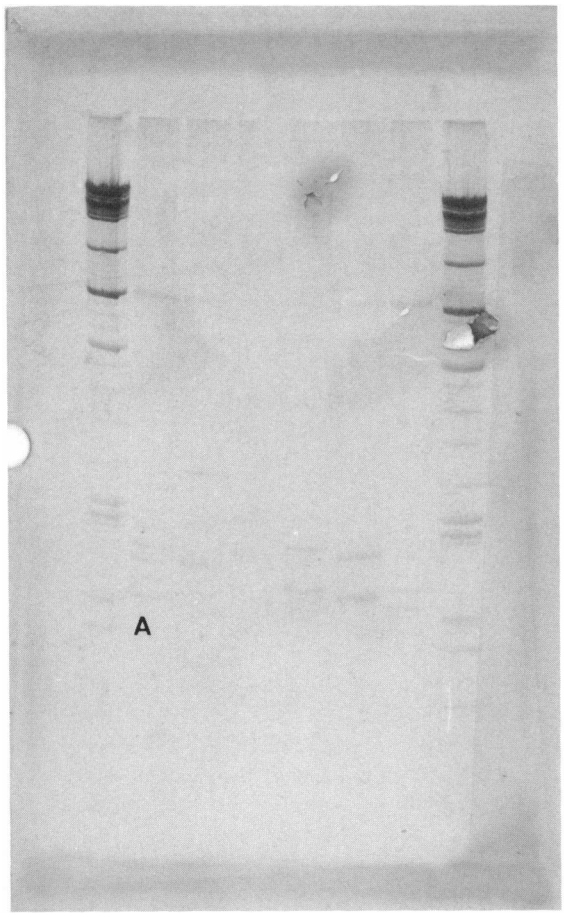

Figure 4 Silver-stained AMP-FLP gel used to generate the autoradiogram in fig. 3. Although the patterns are weak, there is sufficient residual DNA contained within the polyacrylamide gel for detection by silver staining.

bromide staining). The technique makes it possible to obtain discrete allelic data and to correctly genotype VNTR profiles for D1S80. Therefore, the conventional approaches for establishing goodness of fit of the phenotype distributions in population sample can be applied with confidence.

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