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Detection of human DNA polymorphisms with a simplified denaturing gradient gel electrophoresis technique

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ABSTRACT **Single base pair differences between otherwise identical DNA molecules can result in altered melting behavior detectable by denaturing gradient gel electrophoresis. We have developed a simplified procedure for using denaturing gradient gel electrophoresis to detect base pair changes in genomic DNA. Genomic DNA is digested with restriction enzymes and hybridized in solution to labeled single-stranded probe DNA. The excess probe is then hybridized to complementary phage M13 template DNA, and the reaction mixture is electrophoresed on a denaturing gradient gel. Only the genomic DNA·probe hybrids migrate into the gel. Differences in hybrid mobility on the gel indicate base pair changes in the genomic DNA. We have used this technique to identify two polymorphic sites within a 1.2-kilobase region of human chromosome 20. This approach should greatly facilitate the identification of DNA polymorphisms useful for gene linkage studies and the diagnosis of genetic diseases.**

The ability to identify DNA sequence heterogeneity by restriction endonuclease analysis has revolutionized gene linkage studies and genetic disease diagnosis. However, one significant limitation of this technique is that sequence differences must be present at restriction enzyme recognition sites in order to be identified in most cases. Differences occurring outside the recognition sites cannot be detected, and even large panels of restriction enzymes recognize only a fraction of the sequence heterogeneity that is present.

In contrast, denaturing gradient gel electrophoresis (DGGE) allows detection of any single-base change that occurs within the long stretches of DNA that form early-melting domains (see ref. 1 for a review of DGGE). An analysis of predicted melting domain structure for a 35-kilobase (kb) human DNA segment suggests that up to 50–70% of all base pair substitutions should be detectable (2). In the DGGE procedure, DNA molecules are held at the temperature of incipient denaturation and electrophoresed through a polyacrylamide gel containing a gradient of increasing concentrations of urea and formamide. DNA fragments moving through the gel eventually encounter a concentration of denaturants where the early melting domain becomes unstable and abruptly melts, producing molecules that are partially double-stranded and partially disordered. These partially melted molecules migrate more slowly, if at all, through the gel. The position in the gradient at which migration becomes altered is unique and characteristic for each individual fragment. Remarkably, molecules that differ by only a single base pair in the early-melting domain can be separated on these gels because of the alteration in domain melting temperature.

Recently Myers *et al.* reported that DGGE could be used to examine known single-base mutations in the globin genes of patients with β-thalassemia (3). Here we show that DGGE can be used to detect previously unidentified genomic DNA polymorphisms. We also describe a simple technique for reducing probe background that improves the quality of the gels and makes the procedure easier to use. Using this simplified method, we demonstrate two polymorphic sites within a single 1.2-kb region of chromosome 20 and show that DGGE can be applied routinely for the screening of human DNA polymorphisms.

MATERIALS AND METHODS

Materials. D20S4, a 1.2-kb *Hin*dIII fragment derived from human chromosome 20 (4, 5), was obtained in a recombinant pBR322 plasmid from Peter O'Connell (University of Utah). The probe recognizes a two-allele *Msp* I restriction fragment length polymorphism (RFLP). Restriction enzymes were obtained from New England Biolabs; DNA polymerase I, large fragment, was from Bethesda Research Laboratories; proteinase K was from Boehringer Mannheim; and radiochemicals were from New England Nuclear. An oligonucleotide (ACGTTGTAAAACGACGGCC, phage M13 universal primer) complementary to M13 template DNA in a region just upstream of the polylinker sequence was synthesized by using an Applied Biosystems DNA synthesizer and was kindly provided by Dr. Gene Brown.

DNA Isolation. DNA was isolated from peripheral blood leukocytes by using modified standard procedures (6, 7).

M13 Clones. A 1.1-kb *Eco*RI–*Bam*HI fragment from intron 14 of the human factor VIII gene (8, 9) was ligated (10) into the *Eco*RI–*Bam*HI site of M13mp11 to make subclone mp11.F841. The 1.2-kb *Hin*dIII chromosome 20 fragment from plasmid D20S4 was ligated (10) into the *Hin*dIII site of m13mp10 to make subclone mp10.D20S4.

Probe Synthesis. Single-stranded radiolabeled probes with a specific activity of 2.4×10^8 cpm/μg (Cerenkov) were synthesized by M13 primer extension. M13 universal primer (10 pmol) was annealed to 0.5 μg of template DNA and extended with the Klenow fragment of DNA polymerase in a 15-μl reaction mixture containing 40 μCi of [α-^{32}P]dATP (3000 Ci/mmol; 1 Ci = 37 GBq); 6.7 μM dATP; 13.3 μM dGTP, dCTP, and dTTP; 10 mM $MgCl_2$; 10 mM Tris·HCl (pH 8.0), and 50 mM NaCl. After primer extension, the probe was digested with an enzyme that cuts once downstream from the insertion. The probe was then separated from other extension products and the M13 template by electrophoresis on an alkaline agarose gel (11) and was recovered by electrophoresis onto DEAE-cellulose membrane (NA 45, Schleicher and Schuell) as recommended by the manufacturer. After elution, the probe was precipitated with ethanol, resuspended in a small volume, and passed over a Sephadex G-50 spin column (12) before use.

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Abbreviations: DGGE, denaturing gradient gel electrophoresis; RFLP, restriction fragment length polymorphism.

Hybridization Reaction. Genomic DNA (10 μg) was digested to completion with the appropriate restriction enzyme, extracted once with phenol/chloroform, 1:1 (vol/vol), once with chloroform, and precipitated with ethanol. The DNA was resuspended in 10 mM Tris·HCl/1 mM EDTA, pH 8.0 (TE buffer) and brought to a final volume of 25 μl containing 0.3 M NaCl, 0.1 M Tris·HCl (pH 8.0), and a 10- to 20-fold molar excess of probe (usually 3000–5000 cpm, Cerenkov). The samples were boiled for 3 min, centrifuged briefly, and incubated 10–14 hr at 65°C. In many cases the resulting hybrids were digested with a second restriction enzyme after hybridization was complete: the sample was adjusted to a total volume of 100 μl containing 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 100 μg of bovine serum albumin per ml, and the appropriate NaCl concentration for the restriction enzyme. After the second digestion, the DNA was precipitated with ethanol, resuspended in TE buffer, and adjusted to give a final volume of 25 μl containing 0.3 M NaCl and 0.1 M Tris (pH 8.0). Finally, to reduce background, 10–25 ng of the appropriate M13 template was added (discussed below), the sample was incubated an additional 10 min at 65°C and then promptly cooled. Gel loading buffer was added to give a final concentration of 0.025% bromphenol blue and 1.5% Ficoll type 400, and the samples were electrophoresed on a gradient gel.

Denaturing Gradient Gel Electrophoresis. For most studies, 6.5% polyacrylamide gels with a linear gradient of denaturant [100% denaturant = 7 M urea, 40% (vol/vol) formamide] were poured between glass plates at a thickness of 0.75 mm and with sample wells 6 mm wide. These gels are termed "parallel gradient gels," since the direction of electrophoresis is parallel to the gradient (1). The plates were fixed into an apparatus (Green Mountain Laboratory Supplies, Waltham, MA) forming a small upper cathodal chamber, and the whole assembly was almost completely submerged in a buffer-filled aquarium that functioned as the anode (13). A peristaltic pump circulated buffer between the two chambers. Temperature of the aquarium was maintained at 60°C with a circulating heater. After electrophoresis the gels were stained with ethidium bromide, photographed, and dried. Autoradiography was performed for 18–72 hr at −80°C with Kodak XAR-5 film and Dupont Cronex Lightning Plus intensifying screens.

Mobility profiles of plasmid DNA were studied on "perpendicular gradient gels." Gels with linear gradients of denaturants were poured without sample-well combs and rotated 90°. The sample was applied as a band across the top of the gel and electrophoresed in a direction perpendicular to the gradient (1).

RESULTS

Improved Technique for DGGE with Genomic DNA. We have developed a simplified method for using DGGE to screen genomic DNA for sequence variation. Genomic DNA fragments are hybridized in solution to a labeled single-stranded probe, and the excess probe is removed by a second hybridization step with phage M13 template DNA. The fragments can then be examined directly by electrophoresis on a denaturing gradient gel.

Solution hybridization of genomic DNA with a 10- to 20-fold excess of probe produces detectable hybrids after as little as 1 hr, reaching a maximum level after 8–24 hr of hybridization (data not shown). Under these conditions at least 90–95% of the probe DNA remains in an unhybridized state. Because this free probe may mask the pattern of DNA hybrids on a denaturing gradient gel, we developed a simple method to prevent excess probe from migrating into the gel. After hybridization of the probe with denatured genomic DNA, excess M13 template DNA is added to the reaction to hybridize with the unreacted probe. This reaction mixture is then directly electrophoresed into a gradient gel. Since the M13 template·probe DNA hybrid is a large, predominantly single-stranded molecule (approximately 8 kb), it remains at the top of the gel, while the genomic DNA·probe duplexes migrate unhindered into the gel. Fig. 1 shows an example of hybridization reactions to which increasing amounts of M13 template DNA have been added before gel electrophoresis. Background due to excess probe is greatly reduced by the addition of M13 template DNA, and M13 template·probe DNA hybrids barely enter the gel.

Since our single-stranded probes are made by extension of a universal M13 primer on an M13 template, the primer forms a short single-stranded overhang on genomic DNA·probe duplexes. These small overhangs did not significantly affect the melting behavior of the DNA (data not shown). However, the primer overhang is complementary to the M13 template DNA and is capable of hybridizing to the excess template DNA added to reduce background. This could potentially reduce the signal from the genomic DNA·probe hybrids. We examined the effect of adding increasing amounts of M13 template DNA to hybridization reactions (Fig. 1). As the amount of added template was increased, background due to unhybridized probe was significantly diminished. However, the signal from the genomic DNA·probe DNA duplex containing a small overhang was also decreased, in contrast to

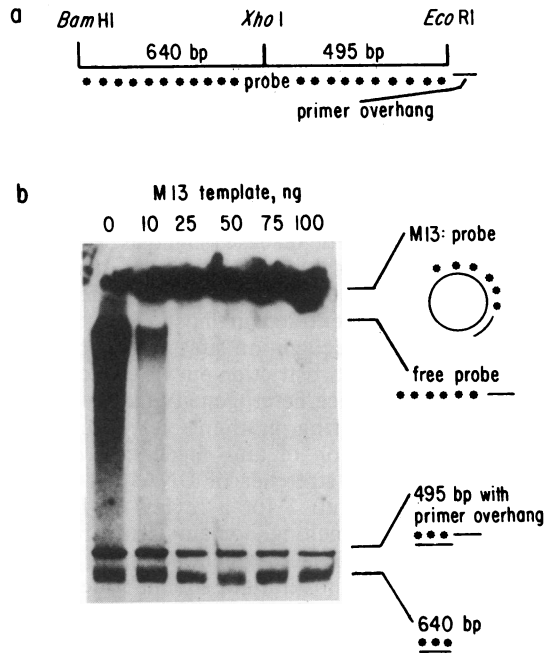

FIG. 1. Addition of phage M13 DNA to reduce probe background. (*a*) A 1.1-kb M13 primer-extended probe (mp11.F841 template) from intron 14 of the human factor VIII gene (8, 9) was hybridized in solution to *Bam*HI/*Eco*RI-digested genomic DNA. The hybrid was digested with *Xho* I to generate a 495-base-pair (bp) *Eco*RI–*Xho* I duplex with a 22-base single-stranded primer overhang and a 640-bp *Bam*HI–*Xho* I duplex with no overhang. (*b*) After ethanol precipitation and resuspension of the DNA in hybridization buffer, M13 template DNA was added as indicated to aliquots containing 10 μg of digested DNA. The samples were hybridized for 10 min at 65°C and electrophoresed on a 6.5% acrylamide/0–50% denaturant gradient gel at 150 V for 3.5 hr. The dried gel was exposed for 18 hr to Kodak XAR-5 film with an intensifying screen at −80°C. Increasing amounts of M13 template hybridized to excess probe and progressively decreased background. The 495-bp genomic DNA·probe duplex with the primer overhang also decreased somewhat, indicating competitive hybridization by the added M13 template; the 640-bp genomic DNA·probe duplex with no primer overhang remained unchanged. Optimal signal-to-background noise was at 10–25 ng of M13 template.

signal from a duplex that contained no overhang. This signal reduction was even more pronounced in duplexes with a longer single-stranded overhang (data not shown). Thus, approximately 10–25 ng of M13 template DNA (mole ratio of template/probe, 80:1 to 200:1) are used in each reaction to obtain optimal background reduction and hybrid band intensity.

General Protocol for Detection of Genomic DNA Polymorphisms by DGGE. The probe D20S4 was examined by DGGE for its ability to detect genomic DNA polymorphisms as part of a gene linkage study using DNA probes from chromosome 20. Although the analysis was applied to a specific case, the sequential approach used in this investigation is generally useful when examining any unsequenced DNA probe for its ability to detect genomic DNA heterogeneity.

We first examined the general melting behavior of the unlabeled 1.2-kb D20S4 probe in a perpendicular gradient gel. This experiment showed that the first domain melted at approximately 30% denaturant. To determine whether a second melting domain could also be studied, we digested the 1.2-kb probe with *Pvu* II, producing two 0.6-kb molecules, fragments A and B (a map is shown in Fig. 4), and repeated the perpendicular gradient gel study with this mixture. Fragment A reached its retardation level at approximately 30% denaturant, indicating that it retained the first melting domain of the 1.2-kb molecule, whereas fragment B was retarded at approximately 37% denaturant, revealing a new melting domain (Fig. 2). This result suggested that both of these melting domains in genomic DNA could be examined for polymorphism on a single gel.

To establish optimum electrophoresis conditions to examine the two domains, a "travel-time" experiment was conducted with unlabeled probe DNA on a parallel gradient gel (Fig. 3). Under the conditions that were used, the first domains of fragments A and B melted after 4–5 hr and after approximately 6 hr of electrophoresis, respectively. This experiment indicated that 8 hr of electrophoresis on a 20–50% gradient gel would ensure that both fragments would be retarded.

To examine fragments A and B in genomic DNA, a labeled single-stranded 1.2-kb probe was synthesized and hybridized to *Hin*dIII DNA digests from individuals in the two study families. After hybridization the mixtures were digested with *Pvu* II to generate the two duplex 0.6-kb fragments A and B. M13 template DNA was added to hybridize the excess probe,

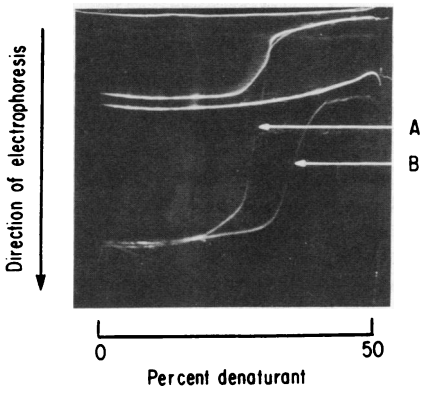

FIG. 2. Melting profiles of D20S4 *Pvu* II fragments. D20S4 plasmid DNA was digested with *Hin*dIII and *Pvu* II, applied across the top of the gel, electrophoresed at 90 V for 8 hr through a perpendicular gradient of denaturant as indicated, and stained with ethidium bromide. A and B are the two D20S4 fragments; the two other bands are from the plasmid vector. Fragment A melts abruptly at approximately 30% denaturant concentration and almost stops moving through the gel; fragment B melts at approximately 37% denaturant but continues to move slowly.

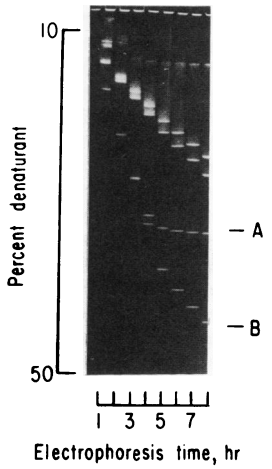

FIG. 3. Separation of D20S4 *Pvu* II fragments with time in a parallel 10–50% denaturant gradient gel. D20S4 plasmid DNA was digested with *Hin*dIII and *Pvu* II. Aliquots were loaded at hourly intervals and electrophoresed at 180 V for the times indicated. At 1–3 hr, fragments A and B comigrated. At 4 hr fragment A had slowed and, between 5–8 hr, advanced very little. Fragment B did not slow until 6 hr, and then continued to advance at a reduced rate. The two prominent slowly migrating bands in the upper part of the gel are from the plasmid vector.

and the mixtures were examined on parallel gradient gels under the conditions described above (Fig. 4).

Characteristics of the Polymorphisms Revealed by Probe D20S4. A series of experiments, carried out with DNA from 41 individuals in the two study families, demonstrated polymorphism in the early melting domains of both fragments A and B. Fragment A showed two common alleles. The farther migrating allele with the higher melting temperature was designated "fast" (*F1*), and the other allele as "slow" (*S1*). Fragment B also showed two common alleles, *F2* and *S2*, as well as a rare, very-early-melting or "very slow" (*V2*) allele (Fig. 4 *b–e*).

The *F* and *S* alleles at both loci segregated in Mendelian fashion in the six nuclear families (including 11 children) tested (Fig. 4*c*). The logs of the relative likelihood of Mendelian inheritance (4) for the loci at fragments A and B were 2.4 and 2.7, respectively. There were three opportunities to examine inheritance of the rare *V2* allele, but in each case the more common complementary allele was inherited.

DNA from each of the 41 study subjects was also examined for the *Msp* I RFLP (4) detected by D20S4 (data not shown). The RFLP alleles are 1.5-kb (fast, *F3*) and 6.5-kb (slow, *S3*). By combining the DGGE and RFLP data with a simple inspection of the relevant pedigrees, haplotypes combining the three loci could be assigned to 30 of these related individuals, representing 60 chromosomes 20. Considering alleles *F1* and *S1* at locus *1* (DGGE fragment A), alleles *F2*, *S2*, and *V2* at locus *2* (DGGE fragment B), and alleles *F3* and *S3* at locus *3* (RFLP *Msp* I), the frequency of each of these haplotypes is: *F1F2F3*, 0.47 (n = 28); *F1V2F3*, 0.03 (n = 2); *S1S2S3*, 0.50 (n = 30). Considering data only from 11 unrelated individuals in this group (spouses), the haplotype frequencies are: *F1F2F3*, 0.41 (n = 9); *F1V2F3*, 0.09 (n = 2); *S1S2S3*, 0.50 (n = 11).

In every case, the *F1*, *F2*, and *F3* (or *V2*, in two cases) alleles segregated concordantly, as did the *S1*, *S2*, and *S3* alleles. This raised the possibility that one or the other of the DGGE polymorphisms might be identical to the *Msp* I polymorphism. Although Southern blots suggested that the polymorphic *Msp* I site was external to the probe sequence, they could not prove that it was not at the extreme end of the probe. We obtained strong evidence that the *Msp* I RFLP and

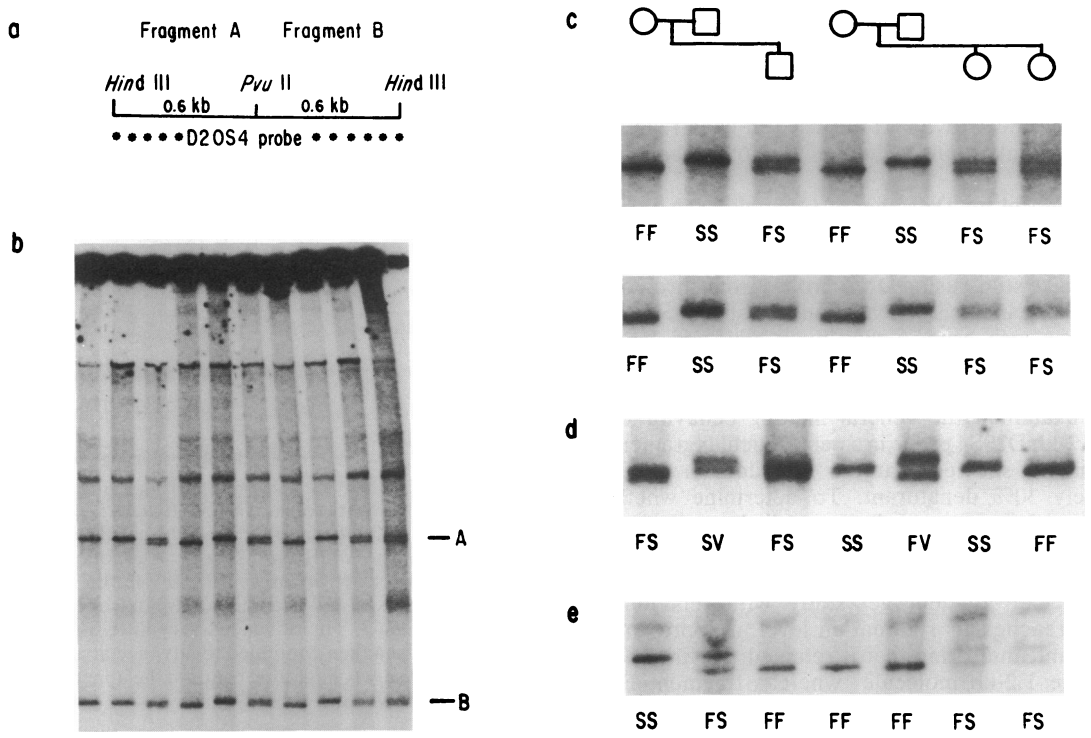

FIG. 4. Characteristics of the D20S4 polymorphisms. (*a*) Diagram of genomic DNA *Hin*dIII fragment·probe hybrid prior to digestion with *Pvu* II. (*b*) *Hin*dIII digests from 10 individuals were hybridized to labeled probe and digested with *Pvu* II to produce fragments A and B as described. These were electrophoresed at 135 V for 10 hr in a 20–50% denaturant gradient gel. Fragments A and B are both polymorphic; this can be seen better in the next figure. (*c*) A closer view of *b* shows the inheritance of *F* (fast) and *S* (slow) alleles of fragments A (*Upper*) and B (*Lower*) in two nuclear families. In this example fragment A alleles are clearly separated; fragment B alleles are close together, producing a broad band in the heterozygous subjects. (*d*) Additional examples of allelic variation in fragment B. The *V* (very slow) allele is present in two individuals. (*e*) Improved resolution of fragment A alleles in a shallower, 25–40% gradient of denaturant.

the DGGE polymorphisms are distinct by demonstrating that digestion of genomic DNA by *Msp* I before DGGE analysis did not change the melting properties of the *F* or *S* alleles of either fragment A or fragment B (data not shown). To settle the question conclusively, however, the DNA region must be sequenced, or other families must be examined to demonstrate that the *F* and *S* DGGE alleles do not always segregate concordantly with the *F* and *S Msp* I RFLP alleles.

Result of Rare *Hin*dIII Site Polymorphism. It was not possible to determine the genotype of one fragment B allele in four individuals (a spouse and three of his children) because of an absence in their DNA of the *Hin*dIII site that delimits the fragment B half of one of the homologous D20S4 sequences (Fig. 4*a*). Because this *Hin*dIII site was absent in one chromosome, the usual 0.6-kb duplex fragment B was not produced by the standard protocol, and its melting behavior could not be observed. A larger, partially duplex molecule must have been produced but could not be recognized on the gel. The *Hin*dIII site polymorphism in these four individuals was confirmed by Southern blot analysis (data not shown).

DISCUSSION

We have described a simplified general procedure for applying DGGE in the screening of human genomic DNA for sequence polymorphisms in regions where the base sequence has not been determined. A probe chosen for testing is first examined on a perpendicular gradient gel to estimate the melting point of its first melting domain. Then a travel-time experiment on a parallel gradient gel is done to establish the conditions that will result in melting of the first domain near the midpoint of the gel. This is an important step, since sequence differences in a melting domain produce altered fragment mobility only when the domain has melted. If this experiment is not done, one might falsely conclude from later experiments that a melting domain in genomic DNA was not polymorphic when, in fact, the domain simply had not melted.

To examine genomic DNA, labeled single-stranded probe is synthesized on an M13 template using a universal primer. The probe is hybridized in solution to genomic DNA that has been digested with the restriction enzyme(s) that define(s) the ends of the probe. The small single-stranded overhang produced by the primer does not interfere in the subsequent analysis. To remove excess probe, M13 template DNA is added prior to electrophoresis; this produces a large hybrid molecule that does not enter the gel and greatly reduces probe background. This procedure eliminates the need to remove excess probe by S1 nuclease digestion (3) and ensures that mobility changes observed on these gels are not due to S1 nuclease-induced artifacts. Electrophoresis is then carried out under the conditions that were defined earlier to produce melting of the first domain of the probe·genomic DNA hybrid near the midpoint of the gel. If sequence differences are present in the first melting domain, bands of differing mobility will be observed after autoradiography. In some cases the probe·genomic DNA hybrid may be digested with another restriction enzyme prior to electrophoresis to yield two or more fragments, allowing analysis of more than one melting domain in a single gel lane using a single probe.

We have used this procedure to examine a 1.2-kb region of chromosome 20 in two extended families. Using probe D20S4, we have identified two polymorphic melting domains, one with two alleles (the *F1* and *S1* alleles of fragment A) and the other with three alleles (the *F2*, *S2*, and *V2* alleles of fragment B). Both regions can be examined together in a single gel, although optimum conditions for each polymor-

phism are established by using slightly different gradients (Fig. 4*d*).

The base sequence differences that produce these two polymorphisms are unknown. Although base methylation can alter melting behavior sufficiently to produce patterns similar to those illustrated here (M.C. and R. Myers, unpublished data), this is not a satisfactory explanation for our findings because the sequence differences we report are inherited in a Mendelian fashion. Insertions or deletions could account for the observed polymorphisms but, if present, would probably be less than 25 bp long, since separation of alleles was never observed in acrylamide gels unless the fragments had melted. These polymorphisms are most likely due to single-base substitutions. RFLP analysis of genomic DNA with D20S4 and at least 16 restriction enzymes has revealed a significant polymorphism only with *Msp* I (4). Our studies show that it is highly unlikely that the *Msp* I RFLP is responsible for either of the DGGE polymorphisms we describe here.

In addition to these two melting polymorphisms, we also identified an additional DNA variant by DGGE that was confirmed as a rare *Hin*dIII site polymorphism. The major significance of this RFLP to our findings is that it interferes with the analysis of one of the melting polymorphisms we describe and exemplifies a potential problem that is general to DGGE analysis: polymorphism of either of the restriction enzyme sites that define the ends of the DNA sequence under analysis may make some samples impossible to examine by DGGE. In such cases the interference may not be immediately apparent and could lead to misinterpretation. For example, absence in one chromosome of one of the usual restriction enzyme sites defining the sequence under study might prevent that allele from being recognized on the gel; the other allele, migrating normally, would be interpreted as a homozygous allele pair. If this problem is suspected, RFLP analysis must also be done.

These findings illustrate the potential value of DGGE for identifying human DNA polymorphisms useful for gene linkage studies and the diagnosis of genetic diseases. The DGGE technique described here has also been used to detect sequence variations in portions of the human factor VIII gene (M.C. and S. Wolf, unpublished data) and in unique random fragments of maize DNA (G. Reidel, personal communication). Given the sensitivity of DGGE for detecting single-base changes, this method seems an attractive alternative to RFLP analysis, particularly if a given probe does not recognize a common RFLP or if an RFLP does not segregate informatively in the population under study. The DGGE approach, alone or in combination with RFLP analysis, should be especially useful where fine resolution linkage maps are being developed.

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