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Genetics

Use of nonisotopic M13 probes for genetic analysis: Application to HLA class II loci

(restriction fragment length polymorphism/insulin-dependent diabetes/DNA tissue typing/biotinylated psoralen/streptavidin-horseradish peroxidase)

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ABSTRACT **Previously, DNA polymorphisms in the HLA gene cluster have been analyzed using radioactive probes in Southern blot experiments; the restriction fragment length polymorphisms (RFLPs) revealed by this analysis are capable of subdividing HLA serological types. Here, we report the use of DNA probes labeled with biotinylated psoralen to provide nonisotopic detection of HLA class II RFLP patterns. These biotinylated probes contain cDNA sequences encoding the α and β chains of DP, DQ, and DR HLA class II genes as inserts in M13 vectors. The recombinant M13 molecules are partially double-stranded with single-stranded HLA cDNA regions and contain biotinylated psoralen covalently linked to duplex DNA by UV irradiation. Following hybridization, the presence of biotinylated probe bound to target DNA is detected using a streptavidin-horseradish peroxidase conjugate, which converts the colorless substrate 3,3′,5,5′-tetramethylbenzidine to a blue precipitate in less than 1 hr. The probe and detection system described here can detect single-copy genes in less than 0.5 μg of total human DNA on Southern blots and generates the same specific RFLP patterns as do probes labeled with ^{32}P by nick-translation. These biotinylated HLA class II probes have been applied to tissue typing for bone marrow transplantation and the study of insulin-dependent diabetes susceptibility, revealing in each case relevant polymorphisms not detected by serologic typing.**

The specificity and sensitivity of DNA hybridization have made possible the application of radioactively labeled DNA probes for the diagnosis of genetic (1) and infectious (2) disease. In addition, specific probes have been used to reveal DNA polymorphisms in the HLA region for use in HLA typing (reviewed in refs. 3 and 4). The HLA region consists of the class I loci, which encode the heavy chain of the classic transplantation antigens, the class II loci, which encode a 33- to 34-kDa α chain and a 28- to 29-kDa β chain that form a cell surface heterodimer, and the class III loci, which encode components of the complement pathway. The HLA class II glycopeptides include three distinct groups of cell surface antigens called HLA-DR, -DQ, and -DP. Both the class I and class II antigens are highly polymorphic cell surface molecules that function as recognition elements for T lymphocytes. Probes derived from the class II genes have identified restriction fragment length polymorphisms (RFLPs) that are correlated with HLA specificities and, in many cases, can subdivide these serologically defined types. These probes have been used for tissue typing as well as for the analysis of disease susceptibility in family and population studies. Based on the high degree of polymorphism revealed by RFLP analysis, another area of potential use for HLA DNA probes is in paternity determination.

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HLA DNA typing requires sensitive probe-detection systems for the analysis of single-copy RFLPs in genomic Southern experiments (5). Following restriction endonuclease digestion, agarose gel electrophoresis, and transfer to a solid support, the target restriction fragment is present in picogram amounts (0.5×10^{-18} mol/μg of total human DNA). Until recently, genomic Southern experiments utilized probes labeled with ^{32}P to specific activities $\geq 10^8$ cpm/μg and autoradiography to detect specific target fragments. We have developed a nonisotopic system that relies on a biotinylated M13 probe and a rapid detection method for use in HLA DNA typing and, more generally, in genomic Southern experiments.

Biotinylated nucleic acid probes for hybridization were first used by Davidson and coworkers (6–8) in applications that did not require the sensitivity needed for Southern experiments. More recently, Ward and coworkers (9) labeled DNA probes with biotin by using DNA polymerase I to incorporate biotinylated nucleotides in the nick-translation reaction. In addition, Ward and colleagues (10) developed a sensitive detection system consisting of a complex of avidin (or streptavidin) and biotinylated alkaline phosphatase, which binds to the biotinylated probe and generates a colored precipitate over the course of several hours. Adaptations of this technology have been used to detect single-copy human genes in Southern experiments (11, 12).

We have developed a DNA probe system, based on the single-stranded bacteriophage M13 (13), a labeling reagent (biotinylated psoralen*), a streptavidin-horseradish peroxidase (HRP) conjugate, and the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (14). The M13 probe is constructed and labeled in two steps. A gapped circle is formed by hybridizing single-stranded M13 DNA containing the desired cDNA insert to linearized M13 replicative form (15) and then is labeled with biotinylated psoralen. After hybridization to the target fragments, the biotinylated probes are detected with streptavidin-HRP which can convert the colorless substrate TMB to a visible blue precipitate in less than 1 hr. A detailed technical discussion of the labeling and detection reagents will be the subject of a subsequent report by J. Sninsky.

MATERIALS AND METHODS

DNA Preparation and Fractionation. Human DNA was purified from tissue culture cells or from blood by standard techniques (16). The cell line WT51, a consanguineous DR4 homozygous typing cell, was kindly provided by J. Bell (Stanford, CA). For the bone marrow transplantation study,

Abbreviations: RFLP, restriction fragment length polymorphism; IDDM, insulin-dependent diabetes mellitus; TMB, 3,3′,5,5′-tetramethylbenzidine; HRP, horseradish peroxidase; kb, kilobase(s).

*Levenson, C. H. & Sheldon, E. (1984) 25th Annual Meeting of the American Society of Pharmacognosy, Aug. 21, 1984, Austin, TX.

DNA was extracted from HLA-typed blood samples that were generously provided by G. Nepom (Virginia Mason Research Center, Seattle, WA) and J. Hansen (Fred Hutchinson Cancer Research Center, Seattle, WA). DNA from a cell line with a homozygous deletion spanning most of the HLA class II region (LCL.174) (17, 18) was kindly provided by R. DeMars (University of Wisconsin, Madison). For the study of the transmission of insulin-dependent diabetes mellitus (IDDM), DNA was extracted from Epstein–Barr virus-transformed lymphocytes from the parents and five of the six children from family 4 whose HLA serotypes were reported by Stetler *et al.* (19). Digestion with restriction endonucleases was performed as described by Maniatis *et al.* (20). See-Quence biotinylated bacteriophage λ *Bst*EII fragments were used as molecular weight standards (Cetus, Emeryville, CA). The DNA was fractionated by electrophoresis in 1% agarose with 0.04 M Tris-acetate/2 mM EDTA, pH 8.0, using the Aquebogue (Aquebogue, NY) 8.5 × 5.5-cm^2 apparatus or the Bio-Rad 10 × 15-cm^2 apparatus at 5 V/cm (21). Blotting to a Genatran 45 nylon membrane (Plasco, Woburn, MA) was carried out for 3–16 hr, using 5× SSPE, as described by Maniatis *et al.* (21). (20× SSPE is 3.6 M NaCl/200 mM NaH_2PO_4/20 mM EDTA, pH 7.4.)

HLA Class II cDNA Probes. Both the nonisotopically labeled See-Quence M13 probes (Cetus, Emeryville, CA) and the probes labeled with ^{32}P by nick-translation were derived from cDNA libraries made from the DR1 homozygous typing cell line LG2 (22, 23) or from the DR1 cell line CA-SC (16). The sequence of the DP α-chain (DPα) cDNA clone (3) and the sequence of the DR β-chain (DRβ) cDNA clone (23) have been reported. All other sequences are available upon request from Cetus. Cloning into plasmids and into M13 vectors was done by standard methods (13, 20). All of the cDNA inserts are >1 kilobase (kb) long and contain all coding sequences of the mature protein.

Gapped-circle DNA probes were prepared by hybridizing the single-stranded M13 DNA containing the desired cDNA insert to *Bam*HI-linearized M13 replication form, essentially as described (15). The resulting probes were photolabeled with a biotinylated psoralen derivative, *N*-biotinyl-*N'*-(4'-methylene trioxsalen)-3,6,9-trioxaundecane-1,11-diamine, which was synthesized as described (24). To monitor incorporation in some experiments, a tritium-labeled biotinylated psoralen derivative was synthesized using a tritiated biotin *N*-hydroxysuccinimide ester (Amersham). Gapped-circle DNA and biotinylated psoralen were combined at 100 μg/ml and 60 μM, respectively (a ratio of 2.5 base pairs of double-stranded DNA to 1.0 biotinylated psoralen derivative), in 10 mM Tris/1.0 mM EDTA, pH 8.0, and irradiated with 360-nm ultraviolet light at 30 mW/cm^2 for 10 min. The labeled gapped circles were precipitated with ethanol and resuspended in 10 mM Tris/1.0 mM EDTA, pH 8.0. This resulted in probes labeled with 5–10 biotinylated psoralen moieties per 100 base pairs of double-stranded DNA, as determined by measuring the absorbance at 333 nm (25) and using a standard curve relating optical density and the incorporation of [^{3}H]biotinylated psoralen (ref. 24; R. Snead, personal communication).

Hybridization. The membrane was incubated in a prehybridization mixture [consisting of 5× Denhardt's solution (20), 5× SSPE, 150 μg of denatured herring sperm DNA per ml, 0.5% sodium dodecyl sulfate, 5% sodium dextran sulfate (M_r 500,000), and 50% formamide] at 42°C for 2–6 hr and then drained. For hybridization, See-Quence probe (Cetus) was added (150 ng/ml) to a separate stock of the same mixture, which had been prewarmed to 42°C, and then combined with the membrane for overnight incubation at the same temperature. Following hybridization, the membrane was washed three times in 2× SSPE/0.5% Tween 20 at room temperature and three times in 0.2× SSPE/0.5% Tween 20 at 60°C to remove excess probe. For comparative experiments, plasmid-derived probes containing the same cDNA regions as the biotinylated M13 probes were labeled by nick-translation (20) and hybridized to membranes, using the same hybridization solutions discussed above except that the sodium dextran sulfate concentration was generally increased to 10%. After hybridization to ^{32}P-labeled probes, membranes were washed as described above and detected by autoradiography at −80°C for overnight to several days using Lightning Plus (DuPont) intensifier screens.

Nonisotopic Detection. For enzymatic detection after hybridization with biotinylated See-Quence probes, the membranes were rinsed in buffer A [5% (vol/vol) Triton X-100/2.7 mM KCl/237 mM NaCl/1.5 mM KH_2PO_4/8 mM Na_2HPO_4, pH 7.4], drained, and then incubated for 40 min in the same solution with See-Quence streptavidin-HRP conjugate (Cetus) at 0.3 μg/ml with respect to HRP. Unbound streptavidin HRP was removed by five 5-min washes with buffer A to which 1% (wt/vol) sodium dextran sulfate and 0.15 M 1,1-diethylurea had been added. Next, the membranes were rinsed in TMB (0.1 mg/ml)/5% ethanol/10 mM sodium citrate/10 mM sodium EDTA, pH 5.0. For color development, the membranes were incubated in 0.0014% H_2O_2/TMB (0.1 mg/ml)/5% ethanol/10 mM sodium citrate, 10 mM sodium EDTA, pH 5.0, for 30–60 min and then read immediately. In order to preserve the pattern of colored bands, the membranes were rinsed four times in water (30–60 min per rinse).

RESULTS

Analysis of Sensitivity and Specificity. Hybridization of a gapped-circle M13 derivative labeled with biotinylated psoralen, as well as detection with streptavidin-HRP, is shown schematically in Fig. 1. The specified reaction conditions for TMB oxidation preserve HRP activity for 1–2 hr and render the blue reaction product insoluble (P. Sheridan, R. Goodson, and D. Birch, personal communications). Although we

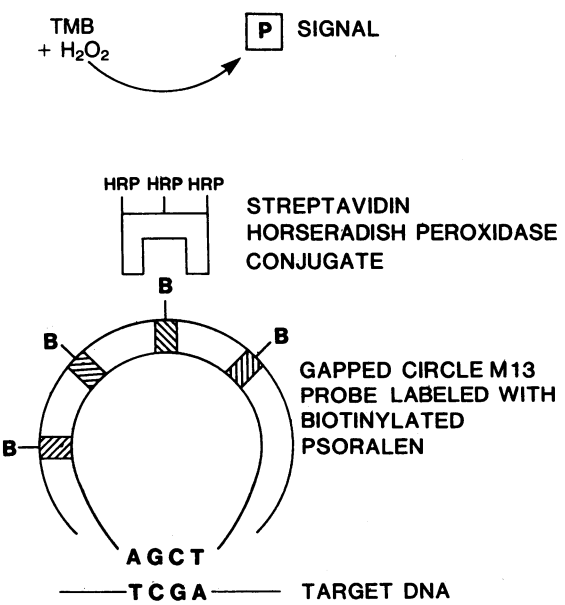

FIG. 1. Nonisotopic probe detection system. The biotinylated M13 probe first hybridizes to the membrane-bound target DNA fragment. After excess probe is washed away, the streptavidin-HRP conjugate is added, which binds the biotin (B) groups on the probe. Next, after excess conjugate is washed away, H_2O_2 and a colorless substrate (TMB) are added and converted to a blue precipitate by the streptavidin-HRP conjugate. Thus, probe hybridized to a target restriction fragment is detected as a blue band on the Southern blot.

have applied this probe-detection system to other DNA targets, the present focus is on the analysis of HLA class II RFLPs.

To establish the sensitivity and specificity of an HLA-DQα M13 probe labeled with biotinylated psoralen, a genomic Southern experiment was performed using a dilution series of *Hin*dIII-digested DNA from DR4 homozygous typing cells (WT51) and DNA from cells homozygous for a deletion of most of the HLA class II region (LCL.174) (17, 18). As shown in Fig. 2, the polymorphic 4.8-kb DQα gene fragment, characteristic of the *HLA-DR4* haplotype (ref. 4, H. Erlich, unpublished data), and the invariant 2.5-kb fragment, derived from the DXα locus (18), can be easily seen in as little as 0.5 μg of total human DNA. A faint 0.8-kb band is also seen in the lanes with 4.0 and 2.0 μg of human DNA. The 0.8-kb band has also been detected by probes labeled with ^{32}P by nick-translation (H. Erlich, unpublished experiment). Bands were not detected in the lane with the LCL.174 deletion DNA.

Comparison with Radioactive Probes. To compare the RFLP patterns generated by these biotinylated probes and by ^{32}P-labeled probes, the same DNA sample was analyzed with the nonisotopically labeled HLA class II probes or with plasmid-derived probes containing the same cDNA inserts labeled with ^{32}P by nick-translation. As shown in Fig. 3, comparable results were obtained. It should be noted, however, that some subtle differences can be discerned between the enzymatically detected band patterns and the autoradiographs. In some cases (e.g., the DRβ results in Fig. 3), multiple, closely spaced but discrete bands are detected enzymatically by the nonisotopic probe system but are visualized as a single band by autoradiography. Shorter autoradiographic exposure allowed resolution of these bands but failed to detect the weaker bands. Another difference is that, in some cases (e.g., DRα, DQβ, and DPβ in Fig. 3), bands detected weakly by the nonisotopically labeled M13 probes are not visible on the corresponding autoradiograph,

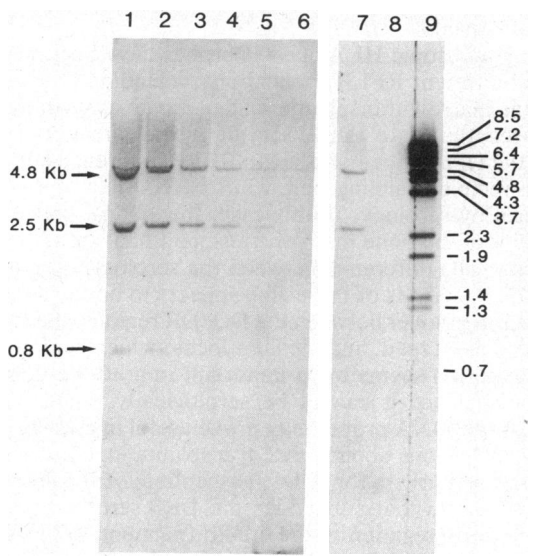

FIG. 2. Detection threshold and specificity of DQα probe. After digestion with *Hin*dIII, 4-, 2-, 1-, 0.5-, 0.25-, 0.125, and 2-μg samples of WT51 DNA (lanes 1–7), and 2 μg of LCL.174 HLA class II deletion DNA (lane 8) were fractionated by agarose minigel electrophoresis, transferred to a Genatran 45 nylon membrane, hybridized to the DQα probe, and detected by the streptavidin-HRP conjugate. For size markers, we included bacteriophage λ DNA digested with *Bst*EII and labeled with biotinylated psoralen (lane 9). Lanes 1–6 and 7–9 were on two separate agarose gels. The expected two bands of 4.8 kb and 2.5 kb can be seen even in the 0.25-μg sample (lane 5). A fainter 0.8-kb band can be seen in the 4.0- and 2.0-μg samples (lanes 1 and 2).

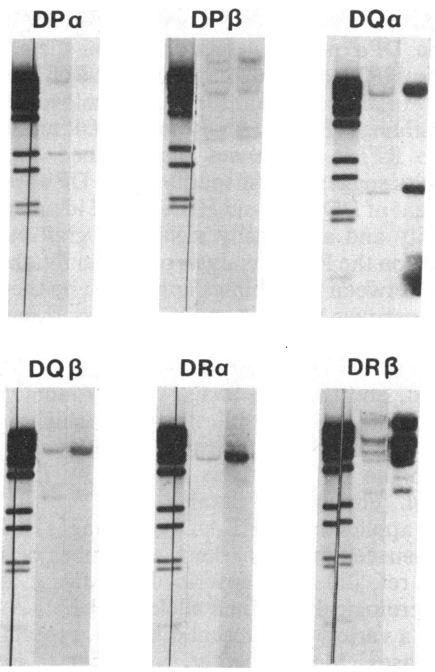

FIG. 3. Comparison of nonradioactive and nick-translated probes. *Bgl* II-digested WT51 DNA (2 μg per lane) was fractionated by agarose minigel electrophoresis and transferred to membranes. Replicate membranes were hybridized to the nonisotopic probes or to DP, DQ, and DR α- and β-chain-specific probes nick-translated by standard procedures (18) to specific activities of 1×10^8 to 9×10^8 cpm/μg. Detection was by color development or autoradiography. In this figure, the blots are grouped in pairs, with the blot detected by color development on the left and the autoradiograph on the right. Autoradiographic exposure was 4 days for DPα, 4 days for DPβ, 16 hr for DQα, 4 days for DQβ, 2 days for DRα, and 4 days for DRβ.

given the indicated exposure times. Longer autoradiographic exposure revealed the presence of these bands. The observed difference in the detection threshold for some fragments may, in part, be attributed to structural differences between nick-translated plasmids and intact M13 probes (see *Discussion*). This structural distinction may also account for the observed difference in the ratio of "strong" to "weak" band intensities (e.g., DQβ and DRα in Fig. 3), since the relative signal intensity of the weak bands is greater if they are detected with our M13 probe system. The enzymatic detection system may also contribute to the reduction in differential band intensities seen with the nonradioactively labeled probes, since band intensity differences are greater in the early stages than at the end of color development.

Genetic Applications. One potential application of the HLA class II nonisotopic probes is in tissue typing for transplantation. Bone marrow transplantation usually requires HLA-matching between donor and recipient for both class I and class II antigens. In some cases, DNA typing may provide clinically relevant information not obtainable from serologic typing. Recently, Marcadet *et al.* (26) have used DNA typing with class I and DQβ probes in making therapeutic decisions regarding bone marrow transplantation for two patients whose lymphocytes expressed so few HLA antigens on the cell surface that serologic methods failed. In addition, Carlsson *et al.* (27) used DNA typing with a DQβ probe to examine bone marrow and kidney donor–recipient pairs among serologically HLA-A,B,C,DR-identical siblings (27).

Using the DPα *Bgl* II polymorphism (22), we have analyzed DNA samples from donor and recipient siblings in a bone marrow transplant case in which the recipient experienced a graft-vs.-host reaction. The donor and recipient siblings were serologically typed as HLA-identical. For DNA typing, a

genomic Southern experiment was performed using the nonisotopic DPα probe; detection of the membrane with streptavidin-HRP revealed that the DPα *Bgl* II RFLP patterns were, in fact, different for these serologically indistinguishable siblings (Fig. 4). Analysis with DR and DQ probes showed no RFLP differences (unpublished experiments). These results suggest that disparity in the DP antigens, even in the context of DQ, DR, and HLA-class I identity, can elicit alloreactivity and a clinically significant graft-vs.-host disease. Based on the RFLP analysis of the family, the observed difference between the siblings appears to be the result of a paternal crossover between the HLA class II loci encoding DR and DQ (A. Amar, G. T. Nepom, E. Mickelson, H.A.E., and J. A. Hansen, unpublished data), which can be serologically typed, and the DPα locus, which cannot. Two other serologically indistinguishable sibpairs in which the recipient experienced graft-vs.-host disease have also proved to be different in the DP region, as revealed by RFLP analysis (Amar *et al.*, unpublished data).

Another application of HLA DNA typing is in the analysis of disease-susceptibility markers. A number of studies (reviewed in ref. 28) have described significant associations between serologically defined alleles at the HLA class I and II loci and a variety of different diseases, especially autoimmune diseases. More recently, a number of investigators have reported polymorphic HLA class II restriction fragments associated with increased risk for IDDM. For example, RFLP analysis using a DRα probe has revealed the association of a specific *Bgl* II fragment with a specific subset of DR3 haplotypes (B8, DR3) increased in IDDM patients relative to controls (19). RFLP analysis of an HLA-typed family with IDDM-affected individuals, showing cosegregation of polymorphic *Bgl* II fragments and DR types, was done using a nonisotopic HLA DRα probe (Fig. 5). The results show that the 4.5-kb band segregates with the DR4 haplotype, the 4.2-kb band segregates with the DR3 and DRw6 haplotypes, and the 3.9-kb band segregates with the DR− haplotype. The IDDM-affected individuals have DR3/DRw6 (4.2 kb/4.2 kb) and DR3/DR4 (4.2 kb/4.5 kb) haplotypes.

DISCUSSION

We have demonstrated that the nonisotopic probe detection system described here is sensitive and specific and can be applied to genomic Southern blot analysis of HLA class II

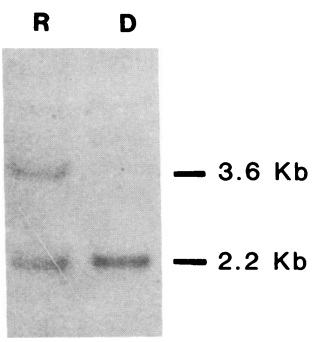

FIG. 4. RFLP analysis of a bone marrow transplant donor–recipient pair. DNA was prepared from cells of HLA-A,B,DR,DQ-identical siblings in which the recipient experienced a graft-vs.-host reaction. HLA serotypes were A1, A2/B8, B40/DR3, DRw6/DQw1, DQw2; complement and glyoxylase were C2C, Bf5, C4A0, C4B1,2 and GLO1,2. For the hybridization experiment, 2 μg of DNA from each individual was digested with *Bgl* II, fractionated by agarose minigel electrophoresis, transferred to a membrane, and hybridized to the nonisotopic HLA-DPα probe. The hybridized probe was detected enzymatically. Lanes: R, recipient; D, donor DNA.

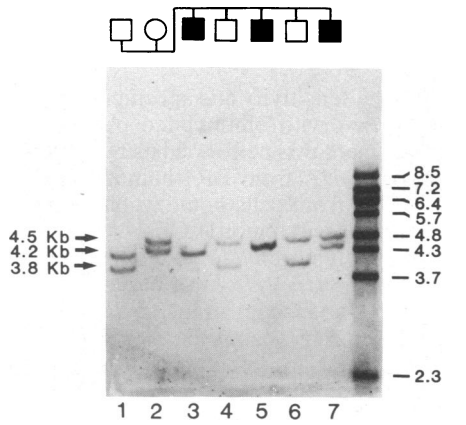

FIG. 5. Segregation of RFLP markers for IDDM within a family with affected members. The father (lane 1) was serologically typed as DR3/DR−, the mother (lane 2) was serologically typed as DR4/DRw6, the sons were serologically typed as DR3/DRw6 (lane 3), DR4/DR− (lane 4), DR3/DRw6 (lane 5), DR4/DR− (lane 6), and DR3/DR4 (lane 7). DNA from an unaffected daughter (DR3/DRw6) was not available for this experiment. For the Southern blot experiment, 2 μg of DNA from the cells of each individual was digested with *Bgl* II, fractionated by agarose minigel electrophoresis, transferred to a membrane, and hybridized to the DRα probe. Hybridization of the probe was detected enzymatically. IDDM-affected individuals are denoted by black boxes. Lane at right shows markers as in Fig. 2.

RFLPs. The detection limit of the nonisotopic DQα probe was 0.25 μg of total human DNA (0.1×10^{-18} mol of the target sequence). In general, concordant results, with some subtle differences, were obtained with the nonradioactive DPα, DPβ, DQα, DQβ, DRα, and DRβ probes and the corresponding probes labeled with ^{32}P by nick-translation. In several of these experiments, the nonisotopic probe system showed higher resolution for most bands and higher signal intensity for weak bands.

The nonisotopic HLA class II probes have been tested in several different RFLP applications, including DNA typing for bone marrow transplantation and the analysis of markers for susceptibility to IDDM. In the bone marrow transplant case, the DPα probe revealed an RFLP difference between a donor–recipient sibling pair whose HLA haplotypes were identical by serology. In this case, the sibling who was the recipient of the bone marrow transplant had a graft-vs.-host reaction. The difference between the serological typing and the RFLP analysis of these sibs appears to be the result of a paternal crossover between the DQ/DR region, which can be serologically typed, and the DPα locus, which cannot. Since the DP region seems to be important in graft-vs.-host reactivity and since it cannot be serologically typed, class II nonisotopic DNA probes may prove useful in making critical clinical decisions about tissue transplantation. In the family analysis of markers for IDDM susceptibility, the DRα *Bgl* II polymorphism that subdivides the DR3 serologic type (19) reveals cosegregation of the 4.2-kb fragment and IDDM. In the application of class II RFLP analysis to paternity determination, we have used the DPα *Bgl* II polymorphism to exclude from potential paternity an A1, B8, DR3 individual not excluded by serologic typing. These data will be reported elsewhere.

The nonisotopic M13 probe detection system described here offers several advantages over probes labeled with radioisotopes. These probes have a storage life of many months at 4°C, can be used without special precautions, and can be detected in less than 3 hr after hybridization. Autoradiography of genomic Southern membranes hybridized with ^{32}P-labeled probes typically requires at least 12 hr of expo-

sure and results in band spreading as exposure time is increased.

M13 probes also have theoretical advantages relative to nick-translated probes. Since the hybridizing regions of M13 probes are single-stranded, they should hybridize to the target DNA more efficiently; the complementary strands of nick-translated probes can self-anneal, thereby reducing hybridization to the target DNA. In addition, M13 probes are at least 7 kb long, whereas nick-translated probes are typically less than 1.0 kb long. Therefore, an M13 probe consisting of vector DNA and an inserted hybridizing region should be able to accommodate more reporter groups (e.g., biotin moieties) than nick-translated probes. Another advantage of M13 probes, as described here, is that they contain the intact hybridizing region in every probe molecule; therefore, all of the probe molecules can hybridize to restriction fragments that contain different segments of the target sequence. In contrast, nick-translated probes consist of a heterogeneous distribution of molecules containing different segments of the sequence homologous to the target. Therefore, the proportion of probe molecules that can hybridize to a genomic restriction fragment (and, thus, the relative signal intensity) depends on the extent of the sequence within the genomic restriction fragment that is homologous to the probe. Since the M13 probes are less sensitive to target size, restriction fragments containing short target DNA sequences are more likely to be detected by M13 probes than by nick-translated probes.

The high sensitivity of the streptavidin-HRP detection system is thought to be based on two features. First, TMB is oxidized more rapidly with less catalytic inactivation of the enzyme than are the commonly used alternative substrates, such as diaminobenzidine. Second, the cationic TMB oxidation product is efficiently immobilized by dextran sulfate adsorbed to the solid support. Membrane loading of the polyanion dextran sulfate is improved by use of a cationic nylon for Southern blotting. The few other HRP substrates that generate insoluble colored products are insufficiently sensitive to achieve the detection limits needed for RFLP analysis of the human genome (P. Sheridan, R. Goodson, and D. Birch, personal communications). An additional advantage of TMB as a substrate is its well-documented lack of carcinogenicity or mutagenicity (29–32).

In general, the sensitivity of this probe detection system is thought to result from the large number of reporter groups (≈700 biotin moieties per probe molecule) and the high activity of HRP with the substrate TMB. This system promises to be useful for the analysis of HLA DNA polymorphisms (DNA typing) as well as for general genetic analysis of the human genome.

This work was the result of close teamwork of a large technical staff. We acknowledge their many contributions and sharing of data and results prior to publication. We are grateful to Glenn Horn, Teodorica Bugawan, and Carolyn Flugstad for HLA class II gene cloning; Diana Ho and Todd Smith for probe construction and labeling; Richard Snead for contributions to probe photochemistry and hybridization optimization; Lois Aldwin, David Birch, Ross Cox, Robert Goodson, Patrick Sheridan, and Robert Toso for the development of the detection technology including conjugate design and construction, enzymology, and protocol development; and Michael Innis and Robert Bruner for useful discussions. We thank John Sninsky for his many helpful suggestions and for his support during the later phase of the project. We thank Kathy Levenson and Ruth Bengelsdorf for careful manuscript preparation.

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