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Genetics

Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes

(polymerase chain reaction/"reverse dot blots"/nonradioactive detection/*HLA-DQA* locus/β-thalassemia)

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ABSTRACT **The analysis of DNA for the presence of particular mutations or polymorphisms can be readily accomplished by differential hybridization with sequence-specific oligonucleotide probes. The *in vitro* DNA amplification technique, the polymerase chain reaction (PCR), has facilitated the use of these probes by greatly increasing the number of copies of target DNA in the sample prior to hybridization. In a conventional assay with immobilized PCR product and labeled oligonucleotide probes, each probe requires a separate hybridization. Here we describe a method by which one can simultaneously screen a sample for all known allelic variants at an amplified locus. In this format, the oligonucleotides are given homopolymer tails with terminal deoxyribonucleotidyltransferase, spotted onto a nylon membrane, and covalently bound by UV irradiation. Due to their long length, the tails are preferentially bound to the nylon, leaving the oligonucleotide probe free to hybridize. The target segment of the DNA sample to be tested is PCR-amplified with biotinylated primers and then hybridized to the membrane containing the immobilized oligonucleotides under stringent conditions. Hybridization is detected nonradioactively by binding of streptavidin-horseradish peroxidase to the biotinylated DNA, followed by a simple colorimetric reaction. This technique has been applied to *HLA-DQA* genotyping (six types) and to the detection of Mediterranean β-thalassemia mutations (nine alleles).**

Differential hybridization with sequence-specific oligonucleotide probes has become a widely used technique for the detection of genetic mutations and polymorphisms (1–5). When hybridized under the appropriate conditions, these synthetic DNA probes (usually 15–20 bases in length) will anneal to their complementary target sequences in the sample DNA only if they are perfectly matched. In most cases, the destabilizing effect of a single base-pair mismatch is sufficient to prevent the formation of a stable probe–target duplex (6). With an appropriate selection of oligonucleotide probes, the relevant genetic content of a DNA sample can be completely described.

This very powerful method of DNA analysis has been greatly simplified by the *in vitro* DNA-amplification technique, the polymerase chain reaction (PCR) (7–9). The PCR can selectively increase the number of copies of a particular DNA segment in a sample by many orders of magnitude. As a result of this 10^6- to 10^8-fold amplification, more convenient assays and nonradioactive detection methods have become possible (10–12). These PCR-based assays are usually done by amplifying the target segment in the sample to be tested, fixing the amplified DNA onto a series of nylon membranes, and hybridizing each membrane with one of the labeled oligonucleotide probes under stringent hybridization conditions. However, each probe must still be individually hybridized to the amplified DNA and the process can easily become difficult in a system where many different mutations or polymorphisms occur.

One approach to address this procedural difficulty is to "reverse" the DNAs: attach the oligonucleotides to the nylon support and hybridize the amplified sample to the membrane. Thus, in a single hybridization reaction, an entire series of sequences could be analyzed simultaneously. The strategy we adopted was to immobilize the oligonucleotides onto nylon filters by ultraviolet fixation. Exposure to UV light activates thymine bases in DNA, which then covalently couple to the primary amines present in nylon (13). It seemed unlikely, however, that short oligonucleotides could be directly attached to nylon in this manner and still retain their ability to discriminate at the level of a single base-pair mismatch. Consequently, the addition of a long deoxyribothymidine homopolymer tail, poly(dT), to the 3′ end of the oligonucleotide appeared promising for several reasons. First, the poly(dT) tail would be a larger target for UV crosslinking and should preferentially react with the nylon. Second, dTTP is very readily incorporated onto the 3′ ends of oligonucleotides by terminal deoxyribonucleotidyltransferase and would permit the synthesis of very long tails (14). (Deoxyribothymidine would also be the most efficiently incorporated base if a purely synthetic route were chosen.) Third, Collins and Hunsaker (15) had shown that the presence of a poly(dA) homopolymer tail, used to introduce multiple ^{35}S labels, did not affect the function of sequence-specific oligonucleotide probes.

We have used this technique to attach oligonucleotide probes specific for the six major *HLA-DQA* DNA types (16) and the eight most common Mediterranean β-thalassemia mutations (4) to nylon filters. The target segment of the DNA sample to be tested (either *HLA-DQA* or β-globin) was amplified by PCR with biotin-labeled primers to introduce a nonradioactive tag. Hybridization of the amplified product to the immobilized oligonucleotides and binding of streptavidin-horseradish peroxidase conjugate to the biotinylated primers were performed simultaneously. Detection was accomplished by a simple colorimetric reaction involving the enzymatic oxidation of a colorless chromogen that yielded a red color wherever hybridization occurred.

MATERIALS AND METHODS

Tailing of Oligonucleotides. Oligonucleotides were synthesized on a DNA synthesizer (model 8700, Biosearch) with β-cyanoethyl *N*,*N*-diisopropylphosphoramidite nucleosides (American Bionetics, Hayward, CA) by using protocols provided by the manufacturer. Oligonucleotide (200 pmol) was tailed in 100 μl of 100 mM potassium cacodylate/25 mM Tris·HCl/1 mM $CoCl_2$/0.2 mM dithiothreitol, pH 7.6 (17), with 5–160 nmol deoxyribonucleoside triphosphate (dTTP or

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Abbreviation: PCR, polymerase chain reaction.

dCTP) and 60 units (50 pmol) of terminal deoxyribonucleotidyltransferase (Ratliff Biochemicals, Los Alamos, NM) for 60 min at 37°C. Reactions were stopped by addition of 100 μl of 10 mM EDTA. The lengths of the homopolymer tails were controlled by limiting dTTP or dCTP. For example, a nominal tail length of 400 dT residues was obtained by using 80 nmol of dTTP in the above reaction.

Preparation of Filters. The tailed oligonucleotides were diluted into 100 μl of TE (10 mM Tris·HCl/0.1 mM EDTA, pH 8.0) and applied to a nylon membrane (Genetrans-45; Plasco, Woburn, MA) with a spotting manifold (BioDot; BioRad). The damp filters were then placed on TE-soaked paper pads in a UV light box (Stratalinker 1800; Stratagene) and irradiated at 254 nm. Dosage was controlled by the device's internal metering unit. The irradiated membranes were washed in 200 ml of 5× SSPE (1× SSPE is 180 mM NaCl/10 mM NaH_2PO_4/1 mM EDTA, pH 7.2) with 0.5% $NaDodSO_4$ for 30 min at 55°C to remove unbound oligonucleotides. If not used immediately, the filters were rinsed in water, air-dried, and stored at room temperature until needed.

Amplification of DNA. PCR amplification of genomic sequences was performed by a slight modification of previously described procedures (9). DNA (0.1–0.5 μg) was amplified in 100 μl containing 50 mM KCl, 10 mM Tris·HCl (pH 8.4), 1.5 mM $MgCl_2$, 10 μg of gelatin, 200 μM each dATP, dCTP, dGTP, and dTTP, 0.2 μM each biotinylated amplification primer, and 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin–Elmer/Cetus). The cycling reaction was done in a programmable heat block (DNA Thermal Cycler; Perkin–Elmer/Cetus) set to heat at 95°C for 15 sec (denature), cool at 55°C for 15 sec (anneal), and incubate at 72°C for 30 sec (extend) by the "Step-Cycle" program. After 30 repetitions, the samples were incubated an additional 5 min at 72°C. The primers contained a single molecule of biotin attached to the 5′ end of the oligonucleotides (described below).

Hybridization and Detection of Amplified DNA. Each filter with bound oligonucleotides was placed in 4 ml of hybridization solution containing 5× SSPE, 0.5% $NaDodSO_4$, and 400 ng of streptavidin-horseradish peroxidase conjugate (SeeQuence; Eastman Kodak). PCR-amplified DNA (20 μl) was denatured by addition of an equal volume of 400 mM NaOH/10 mM EDTA and added immediately to the hybridization solution, which was then incubated at 55°C for 30 min. (During this incubation, hybridization of PCR product to immobilized oligonucleotide and binding of streptavidin-horseradish peroxidase to biotin present in the PCR product occur simultaneously.) The filters were briefly rinsed twice in 2× SSPE/0.1% $NaDodSO_4$ at room temperature, washed once in 2× SSPE/0.1% $NaDodSO_4$ at 55°C for 10 min, and then briefly rinsed twice in 2× PBS (1× PBS is 137 mM NaCl/2.7 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.4) at room temperature. Color development was performed by incubating the filters in 25–50 ml of red leuco dye (Eastman Kodak) at room temperature for 5–10 min. Photographs were taken for permanent records.

Synthesis of Biotinylated Oligonucleotide Primers. Primary amino groups were introduced at the 5′ termini of the primers by a variation of published procedures (18, 19). In brief, tetraethylene glycol was converted to the monophthalimido derivative by reaction with phthalimide in the presence of triphenylphosphine and diisopropyl azodicarboxylate (20). The monophthalimide was converted to the corresponding β-cyanoethyl diisopropylamino phosphoramidite by standard protocols (21). The resulting phthalimido amidite was added to the 5′ ends of the oligonucleotides during the final cycle of automated DNA synthesis by using standard coupling conditions. During normal deprotection of the DNA (concentrated aqueous ammonia for 5 hr at 55°C), the phthalimido group was converted to a primary amine, which was subsequently acylated with an appropriate biotin active ester. NHS-LC-biotin (Pierce) was selected for its water solubility and lack of steric hindrance. The biotinylation was performed on crude, deprotected oligonucleotide, and the mixture was purified by a combination of gel filtration and reversed-phase HPLC. Additional details of this procedure will be published elsewhere (22).

RESULTS

Binding and Hybridization Efficiency of Tailed Oligonucleotides. The relative efficiencies with which synthetic oligonucleotides with homopolymer tails of various lengths were covalently bound to the nylon filter were measured as a function of UV exposure (Fig. 1 *Left*). Oligonucleotides with longer poly(dT) tails were more readily fixed to the membrane, and all attained their maximum values by 240 mJ/cm^2 of irradiation at 254 nm. In contrast, the $(dC)_{400}$-tailed oligonucleotide required more irradiation to crosslink to the nylon and was not comparable to the equivalent $(dT)_{400}$ construct even after 600 mJ/cm^2 exposure. This difference is consistent with the findings of Church and Gilbert (13) that suggested light-activated thymine bases bind more effectively to nylon than do cytosine bases. The untailed oligonucleotide was also retained by the membrane in a manner that roughly paralleled the poly(dC) product.

Efficient binding of oligonucleotides to the membrane, however, does not necessarily correlate with hybridization efficiency, and so hybridization efficiency as a function of UV dosage was determined in a separate experiment (Fig. 1 *Right*). These results show a distinct optimum of exposure that changes with the length of the poly(dT) tail and is more sharply pronounced for the longer tails. Additional experiments have shown the optimal dosages to be about 20 mJ/cm^2 for the $(dT)_{800}$ and 40 mJ/cm^2 for the $(dT)_{400}$ oligonucleotides (R.K.S., unpublished observations). The peak efficiencies of the $(dT)_{400}$ and $(dT)_{800}$ constructs are around 1% (45–50 fmol of radiolabeled probe annealed to ≈3.5 pmol of tailed oligonucleotide), which is similar to the value reported by Gamper *et al.* (23) for an oligonucleotide probe hybridized to nylon-bound plasmid DNA.

Comparison of the data in Fig. 1 *Left* and *Right* for 60 mJ/cm^2 irradiation indicates that oligonucleotides with longer tails hybridize more effectively than can be accounted for by the additional amounts bound to the filter. This suggests a spacer effect wherein the poly(dT) tails improve hybridization efficiency by increasing the distance between the nylon membrane and the terminal oligonucleotide probe. Besides possible UV damage to the DNA itself, additional exposure causes more of the tail to become attached to the membrane, thus reducing the average spacer length and decreasing hybridization efficiency. The markedly different hybridization profile of the poly(dC) oligonucleotide is compatible with this interpretation. Because cytosines react less efficiently with the filter, hybridization efficiency reaches a plateau where loss due to UV damage and tail shortening are compensated by the fixing of new molecules (see Fig. 1 *Left*). This characteristic of cytosine may make a poly(dC) tail desirable when UV irradiation cannot be carefully controlled. Under the stringent hybridization conditions used in this experiment, no signal was detected for the untailed oligonucleotide.

DNA Typing at the *HLA-DQA* Locus. The *HLA-DQA* test is derived from a PCR-based oligonucleotide typing system that partitions the polymorphic variants at the *DQA* locus into four major DNA types, *DQA1* to *DQA4*, and three *DQA1* subtypes, *DQA1.1* to *DQA1.3* (16). Four oligonucleotides specific for the major *DQA* types, four oligonucleotides that characterize the *DQA1* subtypes, and one control oligonucleotide that hybridizes to all allelic *DQA* sequences (Table

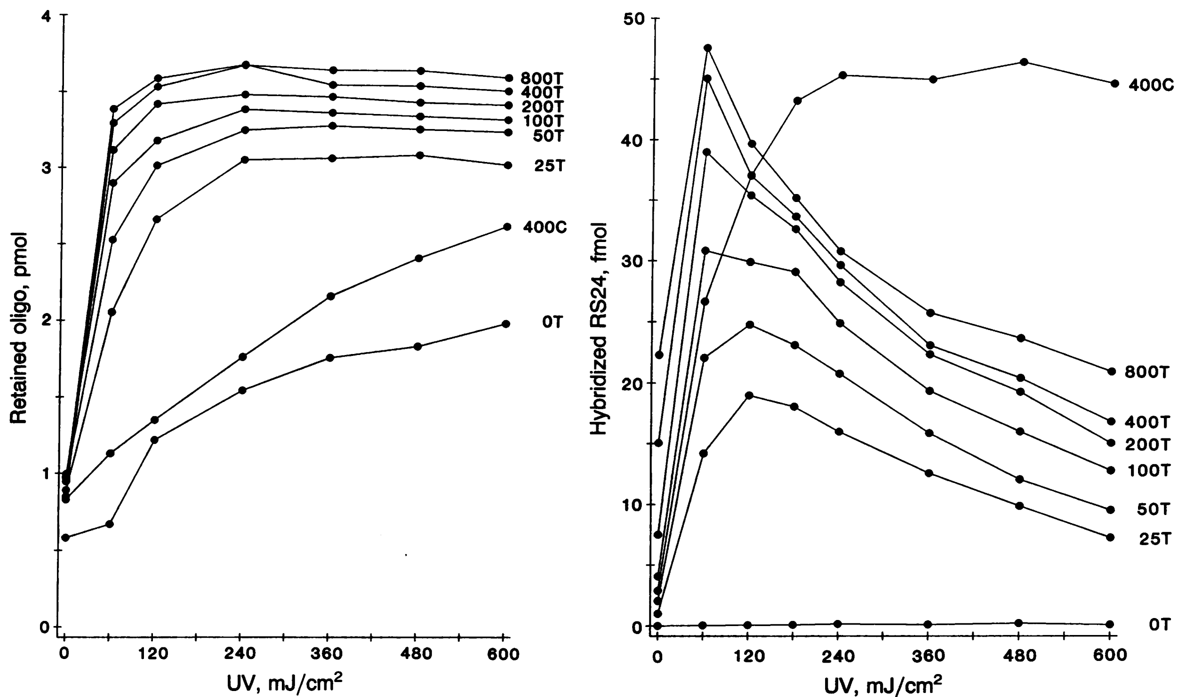

FIG. 1. Filter retention and hybridization efficiency of tailed oligonucleotides as a function of UV dosage and tail length. (*Left*) Filter retention. A 19-base oligonucleotide, 19A (5′-CTCCTGAGGAGAAGTCTGC-3′), was 5′-end-labeled with ^{32}P by using phage T4 polynucleotide kinase and [γ-^{32}P]ATP (10). Portions of the labeled oligonucleotide were given 3′ homopolymer tails with terminal deoxyribonucleotidyltransferase and either dTTP or dCTP. The base compositions and lengths of the tails were as follows: $(dT)_0$, $(dT)_{25}$, $(dT)_{50}$, $(dT)_{100}$, $(dT)_{200}$, $(dT)_{400}$, $(dT)_{800}$, and $(dC)_{400}$. Four picomoles of each oligonucleotide was spotted onto nine duplicate filters, UV irradiated for various times, and washed to remove unbound oligonucleotides; each spot then was measured by scintillation counting to determine the amount crosslinked to the nylon. The values plotted are relative to an unirradiated, unwashed control filter (100% retention). (*Right*) Hybridization efficiency. Filters containing tailed, but unlabeled, 19A were prepared as described above and hybridized under sequence-specific conditions (see *Materials and Methods*) with a ^{32}P-labeled 40-base oligonucleotide, RS24 (5′-CCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGAGTCAG-3′), complementary to 19A. The specific activity of the RS24 was 1.5 μCi/pmol (1 μCi = 37 kBq). Each spot was assayed by scintillation counting. The values plotted are fmol of RS24 hybridized to the membrane.

1) were given 400-base poly(dT) tails and spotted onto nylon filters. The sequence variation that defines the *DQA* types is localized within a relatively small "hypervariable" region of the second exon (24) that can be encompassed within a single 242-base-pair PCR amplification fragment. Biotinylated primers (Table 1) were used to amplify the *DQA* fragment from several genomic DNA samples: six homozygous cell lines and six heterozygous individuals. After hybridization of the amplified DNA to the membranes and color development, the *DQA* genotypes of these samples were readily apparent (Fig. 2).

Although most of the oligonucleotide probes are uniquely specific for one *DQA* type, two of the *DQA1* subtyping probes cross-hybridize to several DNA types. GH89 hybridizes to a sequence common to the *DQA1.2, DQA1.3*, and *DQA4* types, and the probe GH76 detects all *DQA* types except *DQA1.3*. (The latter is needed to distinguish *DQA1.2*/*1.3* heterozygotes from *DQA1.3*/*1.3* homozygotes.) The length and strand specificity of the oligonucleotides were empirically adjusted until their relative hybridization efficiencies and stringency requirements for allelic discrimination were approximately the same. (This was achieved by deter-

Table 1. Sequences of oligonucleotide primers and probes

Name*	Function	Sequence
RS151	*DQA* primer	b-GTGCTGCAGGTGTAAACTTGTACCAG†
RS152	*DQA* primer	b-CACGGATCCGGTAGCAGCGGTAGAGTTG†
RH54 (2)	All *DQA* types	CTACGTGGACCTGGAGAGGAAGGAGACTGCCTG
GH75 (4)	*DQA1* probe	CTCAGGCCACCGCCAGGCA
RH71 (4)	*DQA2* probe	TTCCACAGACTTAGATTTGAC
GH67 (4)	*DQA3* probe	TTCCGCAGATTTAGAAGAT
GH66 (4)	*DQA4* probe	TGTTTGCCTGTTCTCAGAC
GH88 (4)	*DQA1.1* probe	CGTAGAACTCCTCATCTCC
GH89 (4)	*DQA1.2, -1.3, -4*	GATGAGCAGTTCTACGTGG
GH77 (4)	*DQA1.3* probe	CTGGAGAAGAAGGAGAC
GH76 (4)	Not *DQA1.3*	GTCTCCTTCCTCTCCAG

Name*	Function	Sequence
RS151	β-Globin primer	b-ATCACTTAGACCTCACCCTG†
RS152	β-Globin primer	b-GACCTCCCACATTCCCTTTT†
RS187 (8)	Normal $\beta^{1\text{-}110}$	TAGACCAATAGGCAGAGAG
RS188 (8)	Mutant $\beta^{1\text{-}110}$	CTCTCTGCCTATTAGTCTA
RS87 (4)	Normal β^{39}	CCTTGGACCCAGAGGTTCT
RS89 (4)	Mutant β^{39}	AGAACCTCTAGGTCCAAGG
RS189 (0.33)	Normal $\beta^{1\text{-}1,6}$	CTTGATACCAACCTGCCCA‡
RS190 (0.33)	Mutant $\beta^{1\text{-}6}$	TGGGCAGGTTGGCATCAAG
RS191 (1)	Mutant $\beta^{1\text{-}1}$	TGGGCAGATTGGTATCAAG
RS192 (4)	Normal $\beta^{2\text{-}1}$	CCATAGACTCACCCTGAAG
RS193 (4)	Mutant $\beta^{2\text{-}1}$	CTTCAGGATGAGTCTATGG
RS201 (2)	Normal $\beta^{2\text{-}745}$	GCAGAATGGTAGCTGGATT
RS202 (2)	Mutant $\beta^{2\text{-}745}$	GCAGAATGGTACCTGGATT
RS196 (4)	Normal $\beta^{6,8}$	ACTCCTGAGGAGAAGTCTG‡
RS197 (4)	Mutant β^{6}	GACTCCTGGGAGAAGTCTG
RS198 (4)	Mutant β^{8}	TGACTCCTGAGGAGGTCTG

*Where applicable, the values in parentheses indicate the amount (pmol) of tailed oligonucleotide probe applied to the nylon membrane.
†b, Biotin covalently attached to 5′ end.
‡These β-globin oligonucleotide probes each span two sites of potential β-thalassemia mutations and are specific for normal sequences at both positions.

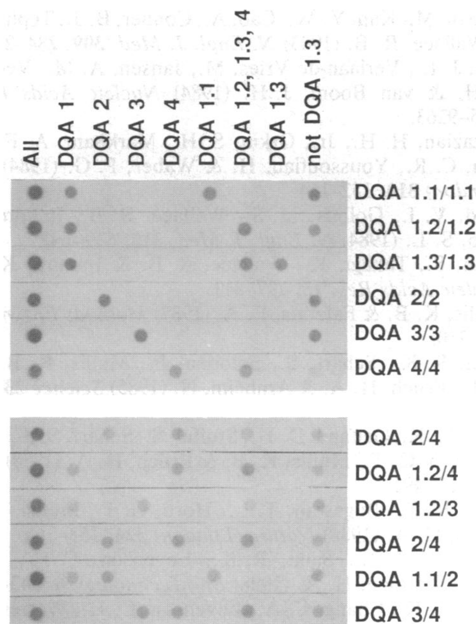

FIG. 2. DNA typing at the *HLA-DQA* locus. Each tailed oligonucleotide probe was spotted onto 12 duplicate membranes, irradiated at 40 mJ/cm^2, hybridized with amplified *DQA* sequences in genomic DNA samples, and treated for color development. The specificity of each immobilized oligonucleotide is given at the top, and the *DQA* genotype of each sample is noted at the right. The name, amount applied to the membrane, specificity, and sequence of each oligonucleotide are listed in Table 1.

mining the optimal hybridization conditions for each member of an initial set of probes, then shortening or lengthening each oligonucleotide until they all hybridized under equivalent conditions.) These eight probes produce a unique hybridization pattern for each of the 21 possible *DQA* diploid combinations.

Detection of β-Thalassemia Mutations. Although there are >54 characterized mutations of the β-globin gene that can give rise to β-thalassemia (25), each ethnic group in which this disease is prevalent has a limited number of common mutations (4, 26, 27). In Mediterranean populations, 8 mutations are responsible for >90% of the β-thalassemia alleles (4). Oligonucleotides were synthesized that are specific for each of these 8 mutations as well as their corresponding normal sequences (Table 1). The oligonucleotides were given $(dT)_{400}$ tails with terminal transferase and applied to membranes. Since the β-thalassemia mutations are distributed throughout the β-globin gene, biotinylated PCR primers that amplify the entire gene in a single 1780-base-pair fragment were used. (This amplification product encompasses *all* known β-thalassemia mutations, not only the predominant Mediterranean mutations examined here.) After hybridization and color development, the β-globin genotypes could be determined by noting the pattern of hybridization (Fig. 3).

Unlike the *DQA* typing system, two oligonucleotide probes are needed to analyze each mutation—one specific for the normal sequence and one specific for the mutant sequence—in order to differentiate normal/mutant heterozygous carriers from mutant/mutant homozygotes. A complicating factor in this analysis is caused by apparent secondary structure in various portions of the relatively long β-globin amplification product that interferes with oligonucleotide hybridization. The relatively high stringency needed to minimize this secondary structure requires the use of longer (e.g., 19-base) oligonucleotide probes. Because this constraint would not permit varying the length of the oligonucleotides to compensate for different hybridization efficiencies, the "balancing" of signal intensities was accomplished by adjusting the amount of each oligonucleotide applied to the membrane. This was done by applying various amounts of each oligonucleotide onto a membrane and then, after hybridization and color development, simply selecting the positive spots that had similar intensity.

DISCUSSION

These studies have demonstrated the feasibility of immobilizing sequence-specific probes onto nylon membranes and hybridizing PCR-amplified, biotin-labeled genomic fragments to the filters to determine the genetic content of the DNA sample. We have applied this method to *HLA-DQA* genotyping and to the detection of β-thalassemia mutations. Although the number of probes used in the two tests were modest (9 for *DQA* and 14 for β-thalassemia), expanding the analyses to include even more oligonucleotides should not be difficult.

The recently described technique of simultaneous amplification of several DNA fragments, "multiplex" PCR (28), should readily permit the concurrent analysis of multiple genetic loci. Using the immobilized-probe format, we have been able to simultaneously amplify and type at three loci: the *Hin*dIII polymorphism in the $^{G}\gamma$-globin gene (29), the *Ava* II

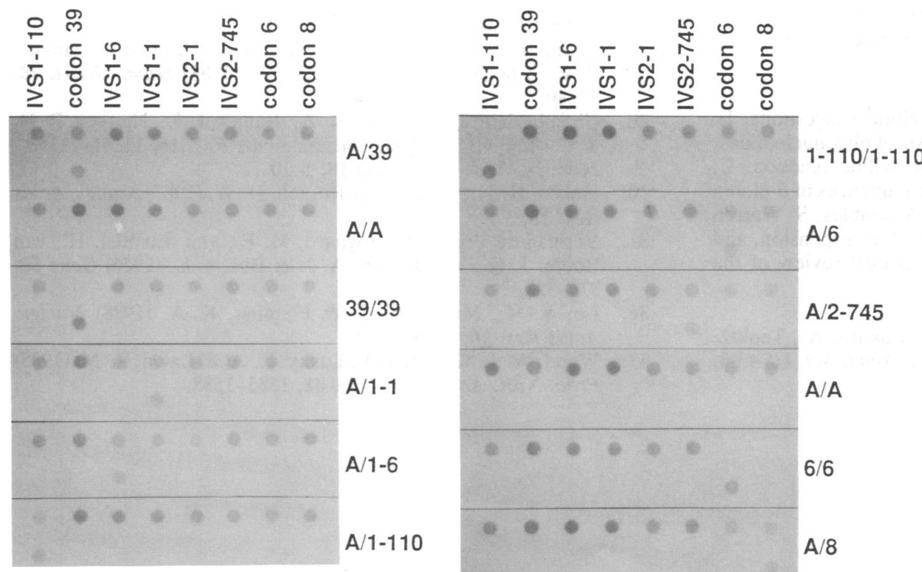

FIG. 3. Detection of β-thalassemia mutations. Various amounts of each tailed oligonucleotide probe were applied to 12 duplicate nylon filters, irradiated at 40 mJ/cm^2, hybridized with amplified β-globin sequences in genomic DNA samples, and treated for color development. The β-thalassemia locus that is detected by each immobilized oligonucleotide pair is given at the top of the filters. For each filter, the upper row contains the oligonucleotide probes that are specific for the normal sequence and the lower row contains the oligonucleotides specific for the mutant sequences. The β-globin genotype of each sample is noted at the right. The name, amount applied to the membrane, specificity, and sequence of each oligonucleotide are listed in Table 1. IVS, intervening sequence (intron).

polymorphism in the low density lipoprotein receptor gene (30), and the *HLA-DQA* gene (R.K.S., unpublished observations). Other genetic targets whose analysis would be simplified by this technique include the detection of somatic mutations in the *RAS* genes, where 6 loci and 66 possible alleles occur (31), some of the HLA class II β-chain genes, where as many as 25 alleles can be detected (T. Bugawan, S. Scharf, and H.A.E., unpublished observations), and β-thalassemia in Middle Eastern populations, where in addition to the endogenous mutations, Mediterranean and Asian Indian mutations are present at significant frequencies (H. Kazazian, personal communication). This format should also prove useful for the detection of infectious pathogens or for environmental surveys of microorganisms by immobilizing a panel of species-specific probes.

The ability to label probes and detect their hybridization without radioactivity is a convenient feature of PCR-based DNA tests and, perhaps more importantly, makes this type of analysis feasible in areas where radioactive labeling reagents are difficult to obtain. In this report, a biotin tag was introduced into the PCR products by means of 5′-biotinylated primers. An alternative labeling strategy based on the incorporation of biotinylated dUTP (32) has also been tried and shown to be very effective (R.K.S., unpublished observations).

One of the prerequisites of this analytical method is that all of the bound oligonucleotides must be sequence-specific under the same hybridization conditions. If necessary, this requirement can probably be met either by adjusting the length, position, and strand specificity of the probes, as was done for the *HLA-DQA* assay, or by varying the amount applied to the membrane, as was done for the β-thalassemia assay. The presence of tetramethylammonium chloride in the hybridization buffer can also serve to minimize the differences among immobilized oligonucleotides caused by varying base compositions (ref. 33; T. Bugawan, personal communication).

Although it may entail some initial effort, the end result is a simple, robust, and potentially automatable system that can be completed (amplification, hybridization, and color development) in 3–4 hr. "Reverse dot blots" should be particularly valuable for assays where the number of potential sequence variations exceeds the number of samples to be tested. Even in situations where the number of samples and probes are approximately equal, the immobilized-probe format may be preferable since many filters can be prepared at one time and stored until needed. To date, this typing system has been used to determine the *HLA-DQA* genotype of >300 unknown samples in forensic and disease-susceptibility studies.

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