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Rapid HLA-DPB typing using enzymatically amplified DNA and nonradioactive sequence-specific oligonucleotide probes

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Abstract. A simple and rapid method for characterizing the polymorphism at the *HLA-DPB1* locus has been developed. The procedure involves the selective amplification of the polymorphic second exon of the *DPB1* locus by the polymerase chain reaction (PCR), followed by hybridization of the amplified DNA with 15 nonisotopic sequence-specific oligonucleotide probes. There are no sequences within the second exon of the *DPB1* locus that uniquely define an allele; rather, each allele appears to arise from the shuffling of a limited number of polymorphic nucleotide sequences in six regions of variability. Consequently, individual alleles are identified by the pattern of hybridization of the 15 probes. Two formats for typing are described. In Format I (the dot-blot), the amplified DNA is ultraviolet (UV) cross-linked to a nylon membrane and hybridized with the oligonucleotide probes which are covalently labeled with horseradish peroxidase (HRP). In Format II (the reverse dot-blot), the oligonucleotides, which have poly-T tails, are bound to the membrane and the immobilized array of probes is hybridized to the PCR product which has incorporated biotinylated primers during the amplification process. In both formats, hybridization is detected by a simple colorimetric reaction. The application of this technology to the fields of tissue typing and individual identity is discussed.

Introduction

The genes encoding the HLA-DPA and -DPB molecules are located at the centromeric end of the *HLA* region on the short arm of human chromosome 6 (Erlich et al. 1986; Hardy et al. 1986) and, perhaps because of their low levels of expression on the cell surface, they were the last of the *HLA-D* genes to be discovered. Serological reagents specific for the DP molecule have been difficult to generate. The DP molecule also elicits a weak response in the primary mixed lymphocyte reaction (MLR; Termijtelen et al. 1984). Consequently, the two methods which have been instrumental in the characterization of the allelic polymorphism at the *DR* and *DQ* loci have proved ineffective for the *DP* loci. The DP molecule does stimulate a strong secondary response in specifically primed T cells (Wank et al. 1978; Mawas et al. 1980; Shaw et al. 1980) and as a result this method, known as primed lymphocyte typing (PLT), has become the standard assay for DP typing. This assay which detects six defined specificities, DPw1–DPw6, can be difficult as well as time-consuming. In addition, cellular (Odum et al. 1987a), biochemical (Lotteau et al. 1987), and restriction fragment length polymorphism (RFLP; Hyldig-Neilsen et al. 1987) analyses suggest that these six specificities are an underestimate of the actual degree of polymorphism within DP. This is not unexpected as approximately 40% of the *DP* alleles cannot be typed in the PLT assay (Baur et al. 1984).

Recent evidence suggests that the DP molecule may be as important as its well-characterized counterparts, DR and DQ, in regulating immune function. The DP molecule has been shown to serve as a restriction element for T-cell recognition of antigen (Eckels et al. 1983; Panina-Bordignon et al. 1989). It may also function as a transplantation antigen since occurrences of acute graft vs host disease (GVHD) in bone marrow transplants of supposed HLA-identical donor-recipient pairs can be correlated with differences in the DP molecule (Amar et al. 1987; Odum et al. 1987b). Finally, the *DP* locus, as defined by the PLT method and RFLP analyses, has also been implicated in the susceptibility to certain autoimmune diseases (Hoffman et al. 1986; Odum et al. 1986; Howell et al. 1988).

The findings have prompted our investigation of the allelic polymorphism at the *DPA1* and *DPB1* loci. Using the polymerase chain reaction (PCR; Saiki et al. 1985;

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Mullis and Faloona 1987) we reported the amplification and sequence analysis of the polymorphic second exons of the *DPA1* and *DPB1* loci from 34 DPw-typed cell lines (Bugawan et al. 1988). Only the two previously characterized alleles of *DPA1* were detected. However, specific *DPB1* sequences were identified for each of the defined DPw1–w6 types, with *B1*-allele subtypes revealed for the DPw2 and DPw4 specificities. An additional four *DPB1* alleles, which did not correlate with any of the T-cell defined specificities (*DPB1* "blanks") were also identified. Together with the two previously reported *DPB1* sequences (Gustafsson et al. 1984; Ando et al. 1986), a total of 14 *DPB1* alleles had been identified, as well as a remarkable patchwork pattern of polymorphism in the second exon of the *DPB1* locus. There are no allele-specific sequences, rather each allele appears to result from the shuffling of a limited number of polymorphic nucleotide sequences in six regions of variability. Based on these initial sequences, a DPB typing system was developed using ^{32}P-labeled sequence-specific oligonucleotides (SSOs) as probes in a simple dot-blot format to analyze DNA amplified from the second exon of the *DPB1* locus (Bugawan et al. 1988; Angelini et al. 1989).

Since the initial report, this assay has been used to characterize the role of the *DPB1* locus in susceptibility to the autoimmune disorders coeliac disease (Bugawan et al. 1988, 1989; Kagnoff et al. 1989), pauciarticular juvenile rheumatoid arthritis (Begovich et al. 1989), adult rheumatoid arthritis (Begovich et al. 1989), and multiple sclerosis (Begovich et al. 1990). The analyses of these patient and control populations have led to the identification of seven additional *DPB1* alleles, as well as to significant improvements in the DPB typing assay. Here we report a detailed description of two different nonradioactive formats for the PCR-based DNA typing of the *DPB1* locus. Both methods use 15 oligonucleotide probes in a dot-blot assay; however, in Format I, the PCR product is bound to the membrane while in Format II, it is the oligonucleotide probes that are immobilized on the membrane. Format I is preferrable for the analysis of large numbers of samples whereas Format II, in which a sample is incubated with all the probes simultaneously, is significantly simpler and faster for the routine analysis of small numbers of samples.

Materials and methods

Format I. In this protocol, the PCR-amplified product is bound to the filter and each filter is hybridized to one of the different nonradioactively labeled typing oligonucleotides, as well as one control oligonucleotide.

Format I: PCR amplification. Between 0.1 and 1 μg genomic DNA is PCR-amplified (Saiki et al. 1985; Mullis and Faloona 1987) in a 200-μl reaction containing 50 m*M* KCl, 10 m*M* Tris-HCl (pH 8.4), 1.5 m*M* $MgCl_2$, 100 μg/ml gelatin, 175 μ*M* each dATP, dCTP, dGTP, and dTTP, 0.50 μ*M* each amplification primer, and 5.0 units *Taq* DNA polymerase (Perkin Elmer-Cetus Instruments, Norwalk, Connecticut). The amplification primers which flank the polymorphic second exon of the *DPB* locus are UG19 (5′GCTGCAGGAGAGTGGCGCCTCC-GCTCAT3′) and UG21 (5′CGGATCCGGCCCAAAGCCCTCAC-TC3′). These primers are more efficient and specific than the previously reported DB01 and DB03 oligonucleotides (Bugawan et al. 1988). The amplification is carried out in a DNA Thermocycler (Perkin Elmer-Cetus Instruments) using a two-step temperature cycle (denaturation: 95 °C for 30 s; annealing and extension: 65 °C for 30 s) for 30 cycles. A "no" DNA control is always included in the amplification to check for product carry-over (Higuchi and Kwok 1989). The efficiency of the amplification is monitored by examining 3 μl of the PCR product on a 3% Nusieve (FMC, Rockland, Maine), 1% agarose gel.

Format I: attachment of the amplified product to filters. Approximately 100 ng (usually 5–10 μl of the PCR reaction) is denatured in 45 μl of 0.4 N NaOH, 25 m*M* ethylenediaminetetraacetate (EDTA) for 10 min at room temperature (replicates of 16 are required for each sample). The membrane [Genetrans-45 (Plasco, Woburn, Massachusetts) or Biodyne (Pall, Glen Cove, New York)] is pre-wet in 2 × saline-sodium phosphate-EDTA (SSPE) or 10 m*M* Tris, 0.1 m*M* EDTA and 50 μl of the denatured DNA sample is applied using a dot-blot apparatus (BioDot, BioRad, Richmond, California). The DNA is ultraviolet (UV) cross-linked to the membrane using a Stratalinker (Stratagene, La Jolla, California) at 50 mJ/cm^2. Unbound DNA is removed by briefly rinsing the membrane in the same solution used to pre-wet the filters.

Format I: sequence-specific probes and hybridization. Figures 1 and 2 show the amino acid and nucleotide sequences of the polymorphic second exons of the 19 *DPB1* alleles which have been identified. [Our sequence analysis of amplified DNA has failed to verify the previously reported *DPB7* and *DPB12* alleles (see below); consequently they have been removed from these lists.] Since there are no allele-specific sequences for *DPB1*, a panel of 15 oligonucleotides corresponding to sequences within four of the six regions of variability (Table 1; Fig. 2) is used for typing and each allele is identified by a diagnostic pattern of probe hybridization (Table 2). Also included is one control oligonucleotide probe which is specific for a nonvariable sequence just 3′ of region *B* and hybridizes to all allelic *DPB1* sequences. This "All" probe is used to control for the amount of amplified DPB1 DNA in the hybridization reaction. Table 1 shows the sequences of the oligonucleotide probes, the amino acids encoded by the specific nucleotide sequences, and the conditions for hybridization. Two sets of hybridization conditions are given for each probe. The first set uses a hybridization solution of SSPE (3 × or 5 ×) and 0.5% sodium dodecyl sulfate (SDS). Hybridization is carried out in a shaking water bath at the indicated temperature for 30–60 min with 1 pmol horseradish peroxidase (HRP)-labeled (Levenson and Chang 1990) probe per ml hybridization solution (8 ml hybridization solution is used per 96-sample filter). Unbound probe is then removed by washing the filters for 10 min in 0.1 × SSPE plus 0.1% SDS at the indicated temperature. Also reported in Table 1 are the conditions for hybridization using tetramethylammonium chloride (TMACl; Wood et al. 1985). This is a simpler procedure because it requires fewer different hybridization conditions; however, TMACl is expensive and somewhat difficult to make up. Hybridization is carried out at the indicated temperature in 3 *M* TMACl, 0.5% SDS, 10 m*M* Tris (pH 7.2), and 0.1 m*M* EDTA as described above. Unbound probe is removed by washing the filters in 3 *M* TMACl, 50 m*M* Tris (pH 7.2), and 2 m*M* EDTA for 10 min at 37 °C and then for 10 min at the indicated stringent temperature.

Format I: detection. Hybridization of the HRP-labeled SSOs to the DNA is detected by using the colorless soluble substrate, tetramethyl benzidine (TMB, Fluka, Ron Kon Koma, New York), which is converted to a blue precipitate by HRP in the presence of hydrogen peroxide. The

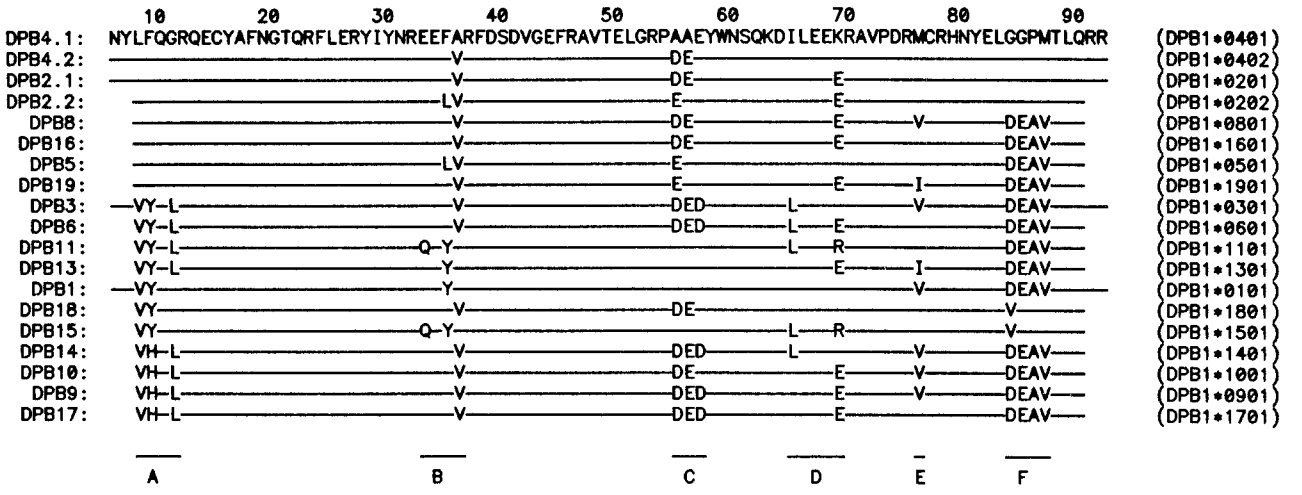


Fig. 1. Alignment of the protein sequences of the first domains of the *HLA-DPB1* genes. The sequences are reported in the one-letter code and aligned with the *DPB1*0401* allele. A *dash* indicates identity with the *DPB1*0401* sequence, and a *space* indicates the sequence was not determined. *Numbers above* indicate the amino acid position in the mature protein, whereas the six regions of variability (*A–F*) are indicated below. The designations to the *right* are those of the official HLA nomenclature committee while those on the *left* are those used in previous publications (Bugawan et al. 1988, 1989; Begovich et al. 1989).

detection procedure is carried out at room temperature with moderate shaking as follows. After washing, the membranes are incubated for 30 min in 1 × Dulbecco's phosphate-buffered saline and then transferred to buffer C (100 m*M* sodium citrate, pH 5) plus 0.1 mg/ml TMB (from a stock solution of 2 mg/ml in 100% ethanol). TMB is precipitated by the immediate addition of hydrogen peroxide to a final concentration of 0.0015% and appears as a blue precipitate; this can take 1–5 min. The reaction can be stopped by transferring the membranes to 0.01 × buffer C. For a permanent record the membranes can be photographed. In addition, the precipitates are stable for over 2 months if stored individually in the dark with buffer C. Alternatively, hybridization of the HRP-labeled probes can be detected using the highly sensitive, commercially available ECL Gene Detection System Kit (Amersham, Arlington Heights, Illinois).

Format II. In Format II, the 15 oligonucleotide probes are bound to the membrane which is then hybridized to the biotinylated PCR product as described (Saiki et al. 1989).

Format II: PCR amplification. The target DNA is amplified as described above except that each PCR primer contains a single biotin molecule attached at the 5′ end of the oligonucleotide as previously described (Levenson and Chang 1990; Saiki et al. 1989).

Format II: attachment of the oligonucleotides to the filters. The 15 oligonucleotide probes (Table 3) are given poly-T tails using either terminal deoxyribonucleotidyltransferase or chemical synthesis and bound to the filters [Genetrans (Plasco)] as previously described (Saiki et al. 1989). Due to their length, the tails are preferentially bound to the membrane, leaving the oligonucleotide probe free to hybridize. Unbound oligonucleotide is removed by washing the membranes for at least 30 min in 1 × SSPE, 0.5% SDS at 50 °C.

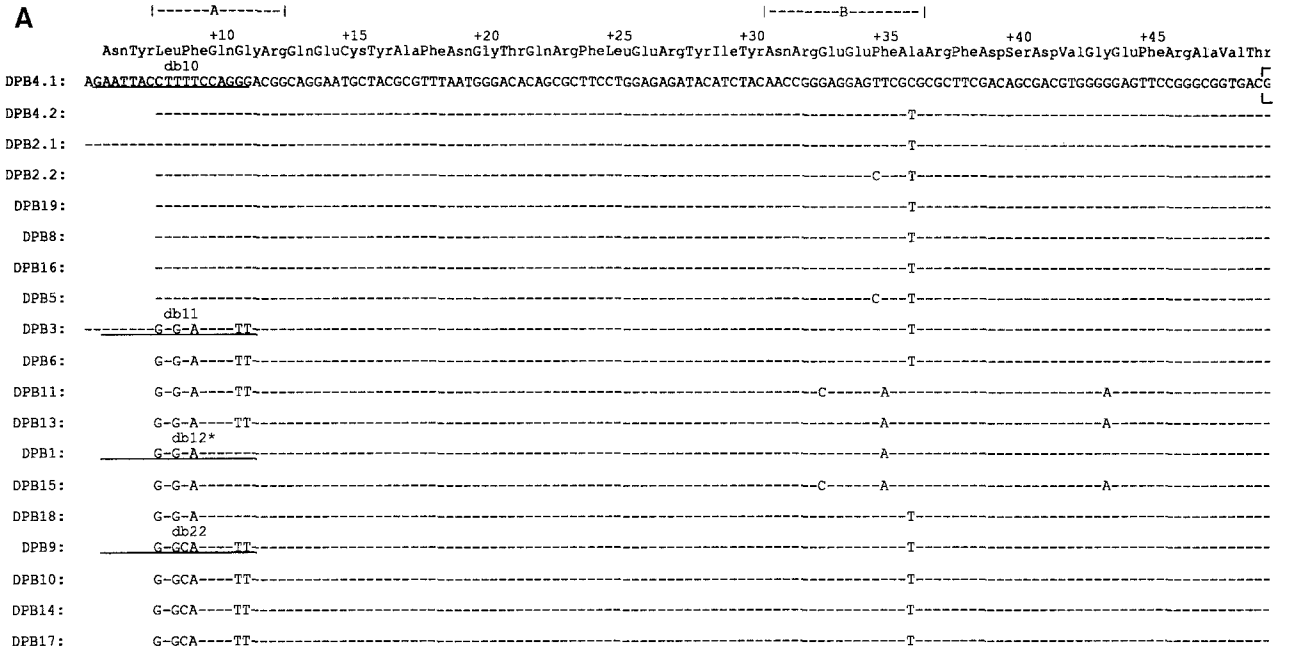
Format II: hybridization and detection. For hybridization, the biotinylated PCR product is denatured at 95 °C for 5 min and then cooled on ice. 50 µl of the PCR product is added to the filters in 2.5 ml prewarmed (50 °C) hybridization solution (1 × SSPE, 0.5% SDS) along with 70 µl of 20 mg/ml streptavidin-HRP conjugate (AmpliType, Cetus, Emeryville, California). Hybridization is carried out for 30 min at 50 °C in a shaking water bath. Unbound probe is removed by washing the filters for 10 min in 0.25 × SSPE, 0.1% SDS at 42 °C in a shaking water bath. Hybridization is detected as described in Format I.

Format I: Allele-specific amplification. The PCR primers UG19 and DB26 (5′CTGCAGGGTCACGGCCTCGTC3′) are used to selectively amplify those alleles encoding the amino acid sequence DEAV at positions 84 to 87. The amplification profile used is the same as that described for UG19 and UG21 and the resulting amplification product can then be typed in the dot-blot assay.

Cloning and sequencing of the PCR product. 100 µl of the original PCR reaction is extracted twice with 50 µl phenol : chloroform (1 : 1), extracted once with 50 µl chloroform, and dialyzed through a Centricon 30 (Amicon, Danvers, Massachusetts) with 2 ml TE (0.01 *M* Tris, 0.0001 *M* EDTA, pH 8.0) by spinning in a Beckman J17 (Beckman, Palo Alto, California) for 25 min at 5000 rpm. The sample is recovered and the DNA is digested for 3 h with the restriction enzymes *Bam* HI and *Pst* I (New England Biolabs, Beverly, Massachusetts) in a total volume of 200 µl by adding 5 units of each enzyme every hour. (These sites are incorporated into the PCR product via the primers.) After digestion, the sample is phenol : chloroform-extracted once, passed through the same Centricon 30 as described above, and brought to a final volume of 20 µl. Three microliters of the sample is put into a 10-µl ligation with 200 ng *Bam* HI/*Pst* I-cut m13mp18 and ligated overnight at 16 °C with T4 DNA ligase (BRL, Gaithersburg, Maryland). Transformations into *Escherichia coli* JM101 are done according to standard procedure (Maniatis et al. 1982) and positive plaques are selected by hybridization to a DPB cDNA. The cDNA used, pDB117, was isolated from the cell line LG2 which is homozygous for *DPB1*0401* (T. Bugawan and H. Erlich, unpublished results). A minimum of six independent clones are sequenced from each sample by the dideoxy chain termination method (Sanger et al. 1980) using ^{35}S-dATP and Sequenase (US Biochemicals, Cleveland, Ohio).

Results

An example of the DPB assay using Format I, in which the PCR product is immobilized on the membrane, is



shown in Figure 3. The DPB type of each sample was determined by comparing the probe hybridization pattern of each sample to the diagnostic pattern for each allele shown in Table 2. The first six samples (CRK, HAS, TOK, BIN-40, JMOS, and LUY) are included in every assay and serve as specificity controls for each of the probes. As Figure 3 shows, all the probes are highly specific with no evidence of cross-hybridization. The current set of *DPB* probes, however, cannot distinguish the *DPB1*0901* allele from the relatively rare *DPB1*1701* allele (Fig. 3, row 3, TOK; Table 2), nor the *DPB1*0801* allele from the rarer *DPB1*1601* allele (Table 2). Both sets differ by one amino acid at position 76 in the second exon (Fig. 1); *DPB1*0801* and *DPB1*0901* have a valine at this position while *DPB1*1601* and *DPB1*1701* have a methionine. While plans are underway to develop probes that will distinguish these alleles, samples with these ambiguous genotypes are currently cloned and sequenced as described above to determine the actual DPB type. Sequence analysis of PCR-amplified TOK DNA has shown TOK to be homozygous for the *DPB1*0901* allele.

Figure 3 also shows that the *DPB1* genotypes **0201/*0501* and **0402/*1901* (*2.1,5* and *4.2,19*) cannot be distinguished from one another using the primers and probes described above (row 7, sample 158; also see Table 2). Of the 190 *DPB1* genotypes possible with 19 alleles, these two heterozygous combinations are the only ones that cannot be distinguished with the sequence-specific probes alone. This ambiguity can be resolved by carrying out an allele-specific amplification. This procedure uses the PCR primers UG19 and DB26 to selectively amplify those alleles encoding the amino acid sequence DEAV at positions 84–87 (in the case of these two genotypes, *DPB1*0501* or *DPB1*1901*). This amplification product can then be typed in the dot-blot assay. The result of this analysis carried out on sample 158 is shown in the last row of Figure 3 (sample 158 ASA) where the PCR product of an allele-specific amplification clearly types as *DPB1*0501*. Therefore, it can be concluded that sample 158 has the *DPB1* genotype **0201/*0501* (*2.1,5*).

This technology has been used to address a number of different questions. In general, allelic diversity at the *DPB1* locus is characterized by different combinations of a limited number of residues at each polymorphic position. The reports in the literature of two *DPB* alleles, *pDAβ5* (Ando et al. 1986; referred to as *DPB12* here and in Bugawan et al. 1988) and *DPw1b* (Lee et al. 1989) that contained unique codons, were inconsistent with this pattern. Neither of these two alleles has been found in any of the over 600 samples examined in this laboratory to date. In order to verify these sequences, DNA from the cell lines reported in the literature was obtained and the *DPB* alleles amplified and characterized both in the dot-blot assay and by sequence analysis. The reported sequence of the *DPB12* allele comes from the cell line AKIBA and is based on a cDNA clone (Ando et al. 1986). This sequence is identical to *DPB1*0901* except for five nucleotide differences which lead to three amino acid substitutions. All three amino acid differences are located outside the six regions of variability at positions 17, 27, and 79. DNA from the cell line AKIBA typed as either *DPB1*0901* or *DPB1*1701* in the dot-blot assay. This is not unexpected as the probes used would not distinguish *DPB12* from these two alleles; however, sequence

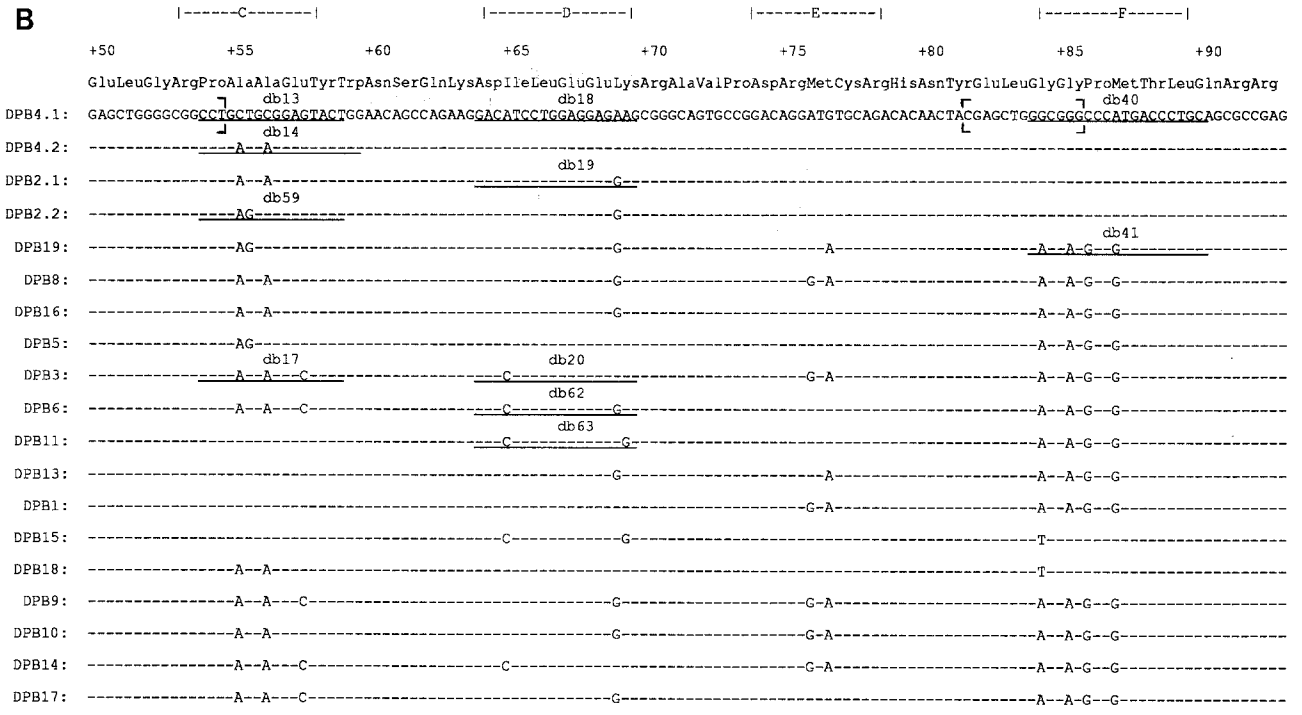


Fig. 2. Alignment of the nucleotide sequences of the first domains of the *HLA-DPB1* genes with the *DPB1*0401* allele. A *dash* indicates identity with the *DPB1*0401* sequence, and a *space* indicates the sequence was not determined. The *underlined* nucleotides and designations *above* correspond to the oligonucleotide probes used in Format I. The amino acid position in the mature protein is indicated *above*, as are the six regions of variability. The putative recombination signals (Wu et al. 1986; Bosch et al. 1989) are shown in *brackets* and contain both a χ-like (GCTGGGG) and minisatellite-like (GGAGGTGGGCAGGARC) sequence.

analysis of six independent clones showed the AKIBA *DPB* sequence to be identical to *DPB1*0901*.

The sequence of two *DPB1* alleles from the cell line RSH (typed by PLT as DPw1) was recently reported (Lee et al. 1989). One allele (*DPw1a*) was identical in sequence to the *DPB1*0101* allele (Fig. 1), the other reported allele (*DPw1b*) had two unique amino acids (three nucleotides) in region *A* but was identical to *DPB1*0402* throughout the rest of exon 2. Analysis of DNA from the RSH cell line available in this laboratory, both with the dot-blot assay and by sequence analysis, showed the RSH line to have the *DPB* alleles *DPB1*0101* and *DPB1*0402*. These reported sequences most likely represent artifacts of either growth in culture, cDNA cloning, or sequencing. We also failed to verify the *DPB7* sequence reported from the RAJI cell line. RAJI is actually homozygous for the *DPB1*0101* allele and, upon checking the literature (Gustafsson et al. 1984), we found that the sequence had been copied incorrectly (Bugawan et al. 1988).

This technology has also been applied to tissue typing for transplantation. We and others have previously reported an analysis of three families in which bone marrow transplants were carried out between presumptive HLA-identical siblings for the treatment of leukemia (Amar et al. 1987). HLA serologic typing had been performed using standard cytotoxicity assays, which do not type for DPB; however, unexpected reactivity in the MLR was observed. In addition, two of the patients survived the transplant long enough to develop acute GVHD. Subsequent RFLP analysis of all three families showed all three donor-recipient pairs to differ in the *DP* region; however, RFLP analysis reveals only that two individuals differ, but does not reveal how they differ, in the protein coding regions.

Individuals from two of these families have now been typed for DPB using Format I. In family UPN2428 (Fig. 4), there appears to be some sort of recombination event either in the donor, the recipient, or both, which includes the *DPB1* locus, such that a *DPB1*0301/DPB1*0401* individual received bone marrow from a *DPB1*0201/DPB1*0401* donor. The *DPB1*0201* and *DPB1*0301* alleles are quite distinct, differing by 11 amino acids in the exon encoding the NH_2-terminal domain (Fig. 1). *DPB1*0201* differs from *DPB1*0401* by four amino acids. In family UPN2441 (Fig. 5), the results of the cytotoxicity assays suggested that the father was *HLA*-homozygous; however, DPB typing showed that he was actually heterozygous, *DPB1*0301/DPB1*0402*. Unfortunately, the sib chosen as the donor carried the paternal chromosome distinct from that found in the recipient so that the *DPB1*0401/DPB1*0402* recipient received bone marrow from a *DPB1*0301/DPB1*0401*

Table 1. Format I SSO probes.

Name	Sequence variant	HRP-SSO probe sequence 5′→3′	Region	Hybridization/wash conditions in SSPE (° C)	Hybridization/wash conditions in TMACl (° C)
DB10	LFQG	GAATTACCTTTTCCAGGGA	*A*	5×, 50°/0.1×, 42° water bath	55°/58°
DB11 (DB58)†	VYQL	ATTACGTGTACCAGTTACG	*A*	3×, 42°/0.1×, 42° air incubator	52°/58°
*DB12**	VYQG	CGTCCCTGGTACACGTAAT	*A*	5×, 50°/0.1×, 42° water bath	55°/58°
DB22 (DB117)	VHQL	ATTACGTGCACCAGTTACG	*A*	3×, 50°/0.1×, 42° water bath	55°/58°
DB13	AAE	CCTGCTGCGGAGTACTG	*C*	3×, 50°/0.1×, 42° water bath	55°/58°
DB14 (DB121)*	DEE	CAGTACTCCTCATCAGG	*C*	5×, 42°/0.1×, 42° water bath	55°/58°
DB59	EAE	CCTGAGGCGGAGTACTG	*C*	5×, 50°/0.1×, 42° water bath	55°/58°
DB17 (DB122)	DED	CCTGATGAGGACTACTG	*C*	5×, 50°/0.1×, 42° air incubator	55°/58°
DB18 (DB72)	ILEEK	GACATCCTGGAGGAGAAGC	*D*	3×, 55°/0.1×, 42° water bath	55°/58°
DB19 (DB73)*	ILEEE	GCTCCTCCTCCAGGATGTC	*D*	5×, 55°/0.1×, 42° water bath	55°/60°
DB20	LLEEK	GACCTCCTGGAGGAGAAGC	*D*	5×, 55°/0.1×, 42° water bath	55°/58°
DB62	LLEEE	GACCTCCTGGAGGAGGAG	*D*	3×, 50°/0.1×, 42° water bath	52°/58°
DB63	LLEER	GACCTCCTGGAGGAGAGG	*D*	3×, 50°/0.1×, 42° water bath	55°/58°
DB40	GGPM	CTGCAGGGTCATGGGCCCCCG	*F*	3×, 50°/0.1×, 42° water bath	55°/58°
DB41	DEAV	CTGCAGGGTCACGGCCTCGTC	*F*	3×, 50°/0.1×, 42° water bath	55°/58°
DB123	"ALL"	CGCTTCGACAGCGACGT		3×, 50°/0.1×, 42° water bath	55°/58°

* The sequence is from the noncoding strand.

† The numbers in parantheses indicate those probes which were redesigned to work under the hybridization conditions for TMACl. The sequences are DB58 (ATTACGTGTACCAGTTA), DB117 (ATTACGTGCACCAGTTAC), DB121 (CAGTACTCCTCATCAG), DB122 (CTGATGAGGACTACTG), DB72 (ACATCCTGGAGGAGAAGC), and DB73 (ACATCCTGGAGGAGGAGC).

Table 2. Determination of *DPB* alleles by oligonucleotide probe hybridization.

DPB1 allele	A				C				D					F	
	LFQG	VYQL	VYQG	VHQL	AAE	DEE	EAE	DED	I-K	I-E	L-K	L-E	L-R	GGPM or VGPM	DEAV
	10	11	12	22	13	14	59	17	18	19	20	62	63	40	41
**0101*	−	−	+	−	+	−	−	−	+	−	−	−	−	−	+
**0201*	+	−	−	−	−	+	−	−	−	+	−	−	−	+	−
**0202*	+	−	−	−	−	−	+	−	−	+	−	−	−	+	−
**0301*	−	+	−	−	−	−	−	+	−	−	+	−	−	−	+
**0401*	+	−	−	−	+	−	−	−	+	−	−	−	−	+	−
**0402*	+	−	−	−	−	+	−	−	+	−	−	−	−	+	−
**0501*	+	−	−	−	−	−	+	−	+	−	−	−	−	−	+
**0601*	−	+	−	−	−	−	−	+	−	−	−	+	−	−	+
**0801*	+	−	−	−	−	+	−	−	−	+	−	−	−	−	+
**0901*	−	−	−	+	−	−	−	+	−	+	−	−	−	−	+
**1001*	−	−	−	+	−	+	−	−	−	+	−	−	−	−	+
**1101*	−	+	−	−	+	−	−	−	−	−	−	−	+	−	+
**1301*	−	+	−	−	+	−	−	−	−	+	−	−	−	−	+
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**1701*	−	−	−	+	−	−	−	+	−	+	−	−	−	−	+
**1801*	−	−	+	−	−	+	−	−	+	−	−	−	−	+	−
**1901*	+	−	−	−	−	−	+	−	−	+	−	−	−	−	+

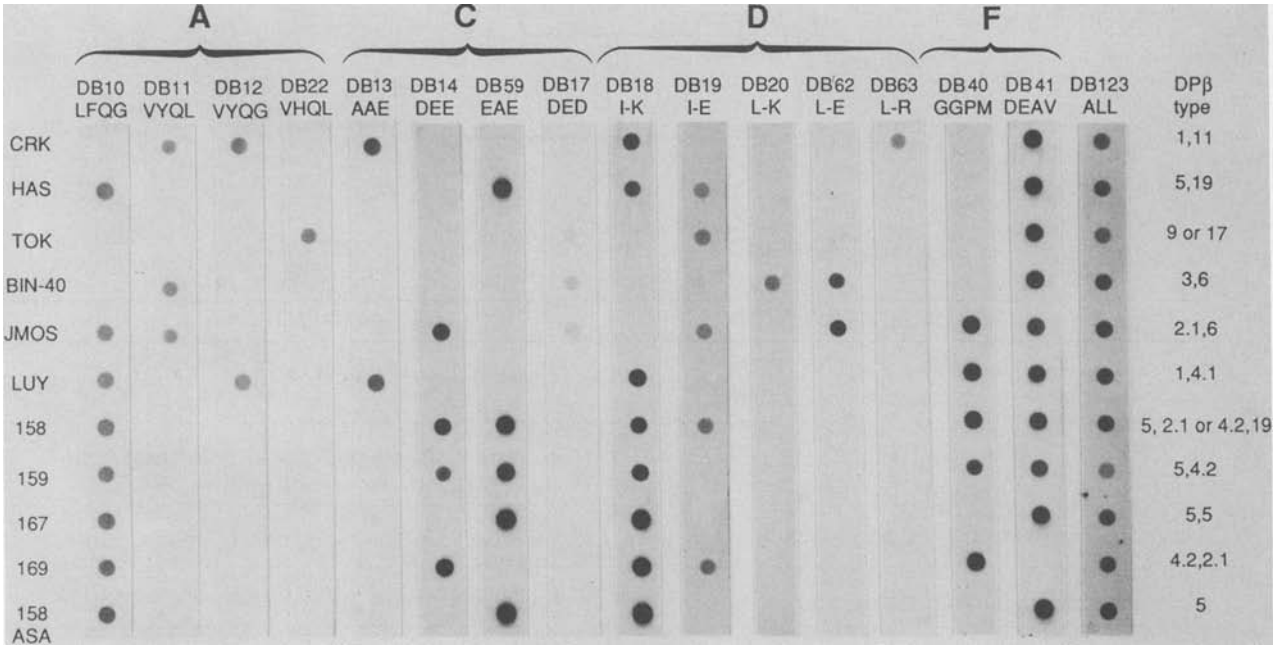


Fig. 3. Format I dot-blot. The sample name and DPB types are listed *on the sides. Along the top* is the probe name as well as the amino acid sequence and region of variability it is specific for. 158 (ASA) is the DPB typing result of an allele-specific amplification of sample 158.

donor. *DPB1*0301* differs from *DPB1*0401* and *DPB1*0402* by 13 and 10 amino acids, respectively, in the NH_2-terminal domain. It should be noted that had a method for DPB typing been available prior to this transplant, sib 3 would have been the preferred donor because she was a perfect HLA match with the recipient.

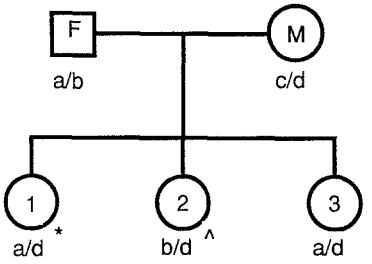
Format I is quite sensitive and very robust; however, each probe must be individually hybridized to the amplified DNA. This process can become cumbersome in a system where many different mutations or polymorphisms occur. This problem has been overcome by the recent development of the reverse dot-blot (Format II; Saiki et al. 1989). In this format, all the probes are given homopolymer tails by using terminal transferase or during chemical synthesis and cross-linked by UV to the membrane. The PCR product is labeled during the amplification process by the incorporation of biotinylated primers and then hybridized to the membrane containing all the immobilized oligonucleotide probes. Hybridization is detected nonradioactively by binding streptavidin-HRP to the biotinylated DNA, followed by a simple colorimetric reaction.

This technique has been applied to the DPB typing system. Since all 15 probes must hybridize specifically under identical conditions, the length of a few of the probes was changed and all were given poly(dT) tails (Table 3). Figure 6 shows an example of the use of Format II to DPB-type the samples HAS, BIN-40, and TOK. Under the conditions described, all of the probes are specific with no evidence of cross-hybridization. However, the signals for certain probes (e. g., LFQG, probe 1 in row 1) are somewhat weaker than others, indicating that different probes have different hybridization efficiencies.

Discussion

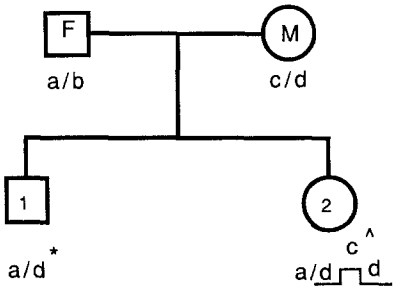
Two formats for a PCR-based DPB typing assay have been described. Both formats use SSOs in a simple, nonradioactive dot-blot format. In Format I, the PCR products are bound to the membrane so that a separate membrane is required for each probe. In Format II, the oligonucleotide probes are bound to the membrane so that a separate membrane is required for each sample. Consequently, the choice of formats will depend both on the numbers are to be routinely typed, Format I would be less which these assays will be done. If very large sample number are to be routinely typed, Format I would be less cumbersome; however, if small sample numbers are to be routinely assayed, Format II would be the preferred assay.

Because of the dispersed and extreme patchwork nature of the polymorphism among the *DPB1* alleles, none of the probes are specific for a given allele, rather they must be used in combination to produce patterns of hybridization which identify specific alleles. The power of this assay not only lies in its speed but also in its ability to accurately identify a large number of different alleles. To date, 19 distinct *DPB1* alleles have been identified and



	PATERNAL HAPLOTYPES	MATERNAL HAPLOTYPES
F:	"a" A1,Cw7,B8,C4A,C4B1,Dw3,DR3,w52,DQw2,**DPB4.2**,GLO-2 "b" A1,Cw7,B8,C4A,C4B1,Dw3,DR3,w52,DQw2, **DPB3** ,GLO-2	
M:		"c" A11,Cw7,B18,C4A3,C4B1,Dw14,DR4,w53,DQw3, **DPB4.1** ,GLO-1 "d" A2,Cw6,Bw57,C4A6,C4B1,Dw11,DR7,w53,DQw3, **DPB4.1** ,GLO-1
1: *	"a" A1,Cw7,B8,C4A,C4B1,Dw3,DR3,w52,DQw2,**DPB4.2**,GLO-2	"d" A2,Cw6,Bw57,C4A6,C4B1,Dw11,DR7,w53,DQw3,**DPB4.1**,GLO-1
2: ^	"b" A1,Cw7,B8,C4A,C4B1,Dw3,DR3,w52,DQw2, **DPB3** ,GLO-2	"d" A2,Cw6,Bw57,C4A6,C4B1,Dw11,DR7,w53,DQw3, **DPB4.1**,GLO-1
3:	"a" A1,Cw7,B8,C4A,C4B1,Dw3,DR3,w52,DQw2,**DPB4.2**,GLO-2	"d" A2,Cw6,Bw57,C4A6,C4B1,Dw11,DR7,w53,DQw3, **DPB4.1**,GLO-1

Fig. 4. Pedigree and possible haplotype assignments of members of family UPN 2428. Without additional family members, the actual maternal haplotypes cannot be determined. The figure outlines one possible interpretation. Alternatively, a double crossover could have occurred in the recipient or different single recombinations could have occurred in each child. *, bone marrow recipient; ^, bone marrow donor; Dw"X", blank or undetected antigen.



	PATERNAL HAPLOTYPES	POSSIBLE MATERNAL HAPLOTYPES
F:	"a" A2,B18,BfS,C4A3,C4B1,Dw5,DR5,w52,DQw3,**DPB4.1**,GLO-1 "b" A30,Bw22,BfF,C4A,C4B31,Dw"X",DR3,w52,DQw2,DPB1,GLO-1	
M:		"c" A3,B35,BfF,C4A3,C4B7,Dw2,DR2,w52,DQw1,**DPB2.1**,GLO-2 "d" A28,Bw53,BfF,C4A3,C4B1,Dw5,DR5,w52,DQw1,**DPB3** ,GLO-1
1: *	"a" A2,B18,BfS,C4A3,C4B1,Dw5,DR5,w52,DQw3,**DPB4.1**,GLO-1	"d" A28,Bw53,BfF,C4A3,C4B1,Dw5,DR5,w52,DQw1,**DPB3** ,GLO-1
2: ^	"a" A2,B18,BfS,C4A3,C4B1,Dw5,DR5,w52,DQw3,**DPB4.1**,GLO-1	"d c d" A28,Bw53,BfF,C4A3,C4B1,Dw5,DR5,w52,DQw 1 DPB2.1 GLO-1

Fig. 5. Pedigree and haplotype assignments of members of family UPN 2441. *, bone marrow recipient; ^, bone marrow donor.

of the 171 possible heterozygous genotypes, there are only 2 [*DPB1*0201/*0501 (2.1,5)* and *DPB1*0402/*1901 (4.2,19)*] which cannot be distinguished in the conventional assay. However, as discussed above, this problem can be overcome using an allele-specific amplification. These results indicate that the *DPB1* locus is as polymorphic as its well-characterized counterparts, *DRB1* and *DQB1*. The sequences of these new alleles also add sup-

Table 3. Format II SSO probes.

Name	Sequence variant	Tailed probe sequence 5′→3′	Region	Hybridization/wash conditions in SSPE (°C)
*DB136**	LFQG	CCGTCCCTGGAAAAGGTAATTC	*A*	1×, 50°/0.25×, 42° water bath
*DB36**	VYQL	CGTAACTGGTACACGTAAT	*A*	1×, 50°/0.25×, 42° water bath
*DB12**	VYQG	CGTCCCTGGTACACGTAAT	*A*	1×, 50°/0.25×, 42° water bath
DB118	VHQL	ATTACGTGCACCAGTTA	*A*	1×, 50°/0.25×, 42° water bath
DB13	AAE	CCTGCTGCGGAGTACTG	*C*	1×, 50°/0.25×, 42° water bath
*DB101**	DEE	CCAGTACTCCTCATCAGGC	*C*	1×, 50°/0.25×, 42° water bath
DB59	EAE	CCTGAGGCGGAGTACTG	*C*	1×, 50°/0.25×, 42° water bath
DB17	DED	CCTGATGAGGACTACTG	*C*	1×, 50°/0.25×, 42° water bath
DB72	ILEEK	ACATCCTGGAGGAGAAGC	*D*	1×, 50°/0.25×, 42° water bath
DB73	ILEEE	ACATCCTGGAGGAGGAGC	*D*	1×, 50°/0.25×, 42° water bath
DB74	LLEEK	ACCTCCTGGAGGAGAAGC	*D*	1×, 50°/0.25×, 42° water bath
DB155†	LLEEE	GACCTCCTGGAGXGAGGAGC	*D*	1×, 50°/0.25×, 42° water bath
DB154†	LLEER	GACCTCCTGXGAGGAGAGGC	*D*	1×, 50°/0.25×, 42° water bath
DB94	GGPM	AGCTGGGCGGGCCCATGAC	*F*	1×, 50°/0.25×, 42° water bath
DB95	DEAV	AGCTGGACGAGGCCGTGAC	*F*	1×, 50°/0.25×, 42° water bath
DB123	ALL	CGCTTCGACAGCGACGT		

* The sequence is from the noncoding strand.
† Inosine (X) was introduced to lower the temperature of disassociation of the probe-target duplex and eliminate cross-hybridization.

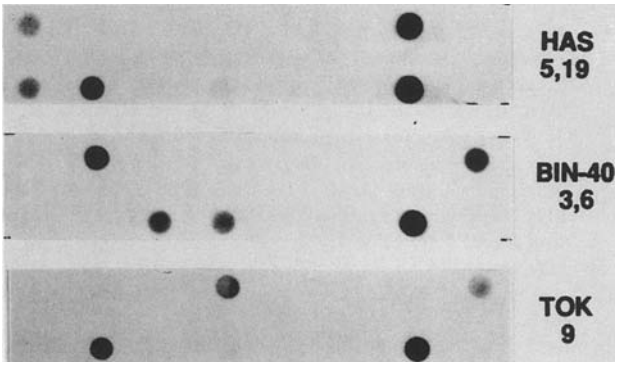


Fig. 6. Format II dot-blot results. The sample name and DPB type are listed to the *right*. The order of the probes bound to the membrane *from left to right* is: row 1, LFQG, VYQL, VYQG, VHQL, AAE, DEE, EAE, DED; row 2, ILEEK, ILEEE, LLEEK, LLEEE, LLEER, GGPM, DEAV.

port to the observation that the polymorphism at the *DPB1* locus appears to result almost exclusively from the shuffling of a limited number of amino acid residues in six regions of variability. This lack of allele-specific sequences is unique to the *DPB1* locus, and suggests that the segmental exchange of DNA sequences between alleles may play a dramatic role in the evolution of polymorphism at this locus. Analysis of the *DPB1* sequences reveals two putative consensus recombination signal sequences around codons 50–54 and 82–85 (Fig. 2), in contrast to the single consensus sequence reported for the second exon of the *DRB1* gene (Wu et al. 1986; Bosch et al. 1989).

This assay has many applications both in basic and clinical research. It has already been used to show that sequences within the DPB molecule may play a role in susceptibility to the autoimmune diseases coeliac disease (Bugawan et al. 1988, 1989; Kagnoff et al. 1989) and pauciarticular juvenile rheumatoid arthritis (Begovich et al. 1989). In addition, this assay has facilitated an extensive study of linkage disequilibrium and recombination with the *HLA* class II region (A. Begovich, G. McClure, R. Helmuth, N. Fildes, V. Suraj, T. Bugawan, H. Erlich, W. Klitz, manuscript in preparation). Perhaps the most important application of this technology will be to the field of tissue typing. Acute GVHD occurs in approximately 35% of supposed HLA-identical donor-recipient pairs (Beatty et al. 1990). This high incidence of GVHD relative to the 1%–3% estimated rate of recombination between DP and the *DR/DQ* region (Termijtelen et al. 1983; Begovich et al. manuscript in preparation) suggests that non-HLA-linked "minor" histocompatibility antigens most likely play a major role in GVHD. However, we (see above) and others (Odum et al. 1987b) have described instances of GVHD which have been correlated with DPB incompatibility. In order to determine whether isolated DP disparity does confer a significant risk for GVHD, a large prospective study of HLA-A, -B, -DR, -DQ identical donor-recipient pairs is currently underway. Given the estimated recombination rate between DP and the *DR/DQ* region and that unrelated individuals are being considered more frequently as donors, DP typing for transplantation may become more important clinically.

Another application of the DPB typing assay may be to the field of individual identity. The large number of alleles at this locus, in addition to the relative absence of linkage disequilibrium between *DPB1* and the remainder of the *HLA-D* region (Begovich et al. manuscript in preparation), makes the combination of the DPB typing assay and another class II typing assay a powerful tool in this field. Whatever the application, the assay described here is a highly sensitive method for typing the *DPB1* locus, because of its speed and its power of discrimination.

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