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Ten *HLA-DR4* alleles defined by sequence polymorphisms within the *DRB1* first domain

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Abstract. We have studied *DRB1* sequence polymorphisms associated with *DR4* subtypes using *DR4*-specific DNA amplification and sequence-specific oligonucleotide probe (SSOP) hybridization. The 5′ amplification primer was designed to hybridize with a unique sequence in the first hypervariable region (HVR) of the *DRB1* second exon of all known *DR4* alleles; the 3′ primer was designed to hybridize with an intron sequence common to all *DRB1* alleles. The specificity of the amplification step was demonstrated by double-blind testing of 105 selected DNA samples. Prospective SSOP typing of *DR4* alleles was performed in 104 unrelated individuals known to be *DR4*-positive, including 13 who were *DR4*-homozygous. A *DRB1* subtype corresponding with the previously defined *DR4*-associated specificities Dw4, Dw10, Dw13.1, Dw13.2, Dw14.1, Dw14.2, Dw15, and DwKT2 could be assigned for each of the 117 *DR4* haplotypes tested. In most cases, *DR4*-homozygous, *DRB1*-heterozygous individuals could be genotyped with the panel of probes. In the course of our analysis, we identified two new *DR4*-related alleles, *DRB1*04.CB* (*DRB1*0410*)[1] and *DRB1*04.EC* (*DRB1*, 0411*)[2] which were recognized by their novel hybridization patterns. The *DRB1* second exon sequence of *DRB1*04.CB* is identical to *DRB1*0405* except at codon 86 where GTG encodes valine instead of GGT encoding glycine. *DRB1*04.EC* is identical to *DRB1*04.CB* except at codon 74 where GAG encodes glutamic acid instead of GCG encoding alanine. Our results provide further evidence that SSOP hybridization is the most effective approach available for subtyping *DR4* haplotypes and identifying unregognized variants. A similar approach should be equally informative for subtyping other *DR* alleles.

Introduction

After the original definition of *DR4* by serologic methods in the Seventh International Histocompatibility Workshop (Bodmer et al. 1978), heterogeneity within the *DR4* family has been demonstrated by cellular, biochemical and DNA techniques (Reinsmoen and Bach 1982; Nepom et al. 1983; Mickelson et al. 1984; Cairns et al. 1985; Gregersen et al. 1987). Using a modified mixed lymphocyte culture assay and HLA homozygous typing cells (HTC), six *DR4* subtypes designated *HLA*-Dw4, w10, w13, w14, w15, and KT2 have been defined (Grosse-Wilde et al. 1984). These *DR4* variants show extensive polymorphism within *DRB1*, each subtype having a unique *DRB1* second exon sequence corresponding to the first domain of the expressed polypeptide. *DRB1* alleles generally demonstrate sequence polymorphism within three hypervariable regions (HVR; Mengle-Gaw and McDevitt 1985). HVR I encompasses the 5′ end of the *DRB1* second exon from approximately codons 9 to 14, HVR II extends from codons 26 to 37, and HVR III extends from codons 67 to 74. The known *DR4* alleles share a common HVR I genomic sequence encoding EQVKHE at codons 9 to 14. Recently, oligonucleotide primers complementary to this shared sequence have been used to achieve selective amplification of *DRB1* second exons from *DR4* haplotypes (Gao et al. 1990; Lanchbury et al. 1990).

We have used allele-specific amplification and sequence-specific oligonucleotide probe (SSOP) methods to study the diversity of *DR4* haplotypes. One goal was to

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned accession numbers M36879 and M55615.

The names *DRB1*0410* and *DRB1*0411* have been officially assigned by the WHO Nomenclature Committee in December 1990. This follows the agreed policy that, subject to the conditions stated in the most recent Nomenclature Report (Bodmer et al. 1990), names will be assigned to new sequences as they are identified. Lists of such new names will be published in the following WHO Nomenclature Report.

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evaluate the conditions under which the DNA amplification step could be made allele-specific. A second goal was to assess the suitability of the SSOP method for genotyping *DR4*-homozygous, *DRB1*-heterozygous individuals, a situation in which two genes are amplified simultaneously by the *DR4*-specific primer pair. Conditions for *DR4*-specific amplification were rigorously tested in a double-blind analysis. With the use of a panel of oligonucleotide probes designed to reflect both consensus and polymorphic sequences, *DR4*-homozygous, *DRB1*- heterozygous samples could be genotyped in nearly all cases through the identification of unique hybridization patterns. In the course of our studies, we identified two novel *DR4* alleles and observed some unusual *DR/DQ* associations. Our results demonstrate the utility of SSOP in typing *DR4* alleles, some of which cannot be assigned by conventional methods, and in identifying previously unrecognized *DR4* variants.

Materials and methods

Cell panel. Initial experiments aimed at determining the optimal polymerase chain reaction (PCR) thermal profile and SSOP hybridization/wash conditions were performed on two panels. The first was a panel of 13 DNA samples selected to represent the haplotypes *DRw10, DR9, DR1,* and *DR7* which differ from *DR4* between nucleotide positions 13–36 of the *DRB1* second exon. These samples included the HLA-homozygous cell lines BSM (DR4, 10W9032), SHY (DRw10), DKB (DR9, 10W9075), HOM2 (DR1, 10W9005), and DBB (DR7, 10W9052). The second was a panel of 100 DNA samples selected to represent *DR1-w18* haplotypes. These samples were selected from 60 *DR4*-negative and 40 *DR4*-positive individuals. In further studies, prospective genotyping for *DR4* alleles was performed on a third panel of DNA samples from 104 individuals who were *DR4*-positive by serological typing. Segregation of *DR4* haplotypes in this group was further analyzed by family studies where possible. In addition to the 104 samples, DNA from 6 DR4-homozygous cell lines MLF (Dw4, 10W9091), YAR (Dw10, 10W9026), JHAF (Dw13, 9W1301), BM92 (Dw14, 10W9092), HAS15 (Dw15, 9W9902), and KT17 (DwKT2, 10W9024) was studied.

HLA typing. Whole blood was processed for serologic, cellular, and DNA studies according to standard methods. For serologic and cellular typing, lymphocytes were isolated from heparinized blood by centrifugation over ficoll-hypaque. Serologic typing for HLA-A, B, C was performed by the standard two stage NIH complement-dependent microcytotoxicity test (Ray 1979). Typing for HLA-DR and DQ was performed using modified Dynabead-purified B cells in a microcytotoxicity assay. HLA-D typing was performed in a modified MLC assay by the use of homozygous typing cells (HTC) as described (Hansen et al. 1986) and HLA-D specificities were assigned using the criteria established in the 8th and 9th International Histocompatibility Workshops (Dupont et al. 1980; Grosse-Wilde et al. 1984).

DNA extraction. DNA was extracted from whole blood collected in ethylene diaminetetraacetate (EDTA) after lysis of erythrocytes and leukocytes according to methods of Marcadet and co-workers (1989). DNA was precipitated from lysed leukocytes with equal volumes of 4M ammonium acetate (Miller et al. 1988) and isopropyl alcohol, washed twice with 70% ethanol, and resuspended in Tris-EDTA (TE). DNA used for sequencing was further purified by phenol-chloroform extraction and precipitation with 3M sodium acetate and ethanol at 70 °C.

DRB1-specific amplification. A 5′ oligonucleotide amplification primer EWP4.1 was originally constructed to nucleotide positions 16–36 of the second exon of *DRB1*; however, when used with the 3′ amplification primer CRX37, amplification could not consistently be achieved. For this reason, the 5′ oligonucleotide primer EWP4.2 was designed to hybridize to nucleotide positions 13–36 for specific amplification of the *DRB1* second exon of *DR4* alleles when used with the CRX37. Oligonucleotide EWP4.2 has the sequence 5′-CCACGTTTCTTG-GAGCAGGTTAAA-3′, and reflects the deduced amino acid sequence PRFLEQVK common to all *DR4* haplotypes. Oligonucleotide CRX37 is an 18-mer that corresponds to a *DRB1* 3′ flanking intron sequence common to all known *DRB1* genes (H. A. Erlich, personal communication; Petersdorf et al. 1990). Human genomic DNA (0.5–1 μg) was amplified by PCR (Saiki et al. 1985; Mullis and Faloona 1987; Saiki et al. 1988) using 50 pmol of each oligonucleotide primer, 2.5 units of *Taq* polymerase, 25 pmol of dNTPs and buffer (GeneAmp Reagent Kit, Cetus, Emeryville, California). For experiments designed to determine the optimal annealing temperature for allele-specific amplification, double-stranded DNA was denatured initially by heating to 94 °C for 7 mins followed by a 35–40 cycle profile using a 1 min denaturation at 94 °C, 2 min annealing at variable temperatures, and 3 min extension at 72 °C. Annealing temperatures tested were as follows: 50, 55, 56, 57, 58, 59, 60, 61, and 65 °C. Presence of the 280 base pair (bp) amplified DNA product was verified by agarose gel electrophoresis. For all subsequent studies a 7 minute denaturation step at 94 °C was followed by a 35–40 cycle 1 min denaturation at 94 °C, 2 min annealing at 60 °C, and 3 min extension at 72 °C.

Oligonucleotide probes. Oligonucleotide probes were designed to be either 15 or 18 nucleotides in length in order to maintain uniform hybridization and wash conditions. The sequences and expected reactivities are described in Table 1. Oligonucleotide probes were synthesized using a DNA synthesizer (Applied Biosystems Model 370A) and desalted over a G-25 Sephadex column (Pharmacia, Piscataway, New Jersey). Probes were 5′ end-labeled with γ-^{32}P ATP using T4 polynucleotide kinase (Boehringer, Indianapolis, Indiana) as previously described (Angelini et al. 1986).

Sequence-specific oligonucleotide probe hybridization. After denaturing with 0.4M NaOH, 25–30 ng of amplified DNA was blotted (Biodot, BioRad, Richmond, California) in duplicate on nylon membranes (Gentran 45, Plasco, Woburn, Massachusetts). After washing with TE, membranes were allowed to air dry completely. Membranes were prehybridized in 3M tetramethylammonium chloride (TMAC) (50 mM Tris HCl pH8, 2mM EDTA, 0.1% SDS, 5 × Denhardt's, 100 mg/ml salmon sperm DNA) for 1 h. ^{32}P-labeled oligonucleotide probes ($1–5 \times 10^6$cpm) were allowed to hybridize for 1–18 h in 3M TMAC at 42 °C. Membranes were then washed twice in 2 × SSPE, 0.1% SDS for 5 minutes at room temperature, once in 3M TMAC buffer (50mM Tris HCl pH8, 2mM EDTA, 0.1% SDS, 3M TMAC; Wood et al. 1985) for 5 minutes at room temperature, and twice in 3M TMAC buffer for 15 min at either 48–49 °C (15-mers) or 58–59 °C (18-mers). Membranes were blotted dry and exposed to Kodak X-OMAT film for 1–18 h.

Sequencing. For sequencing studies, amplified DNA was further purified by separation on a 1.5% low melting point agarose gel followed by phenol-chloroform extraction. Direct 5′ and 3′ sequencing of purified PCR products was performed using a modification of Winship's method (Winship 1989). Independent PCR products from at least three separate amplifications were sequenced. Purified template DNA (100 ng) was mixed with 30 pmol of amplification primer, 5 × Sequenase buffer (40 mM Tris HCl pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl) and 10% dimethylsulfoxide (DMSO). After boiling for 5 min to denature the template and immediately snap-cooling on dry ice, 4.5 μl of labeling mix (0.025 M dithiothreitol, 10 μCi ^{35}S dATP, 2 μl of 1:8 dilution of Sequenase enzyme [US Biochemicals, Cleveland, Ohio] was added to the annealing mixture. The resulting 10.8 μl volume was divided equally into four reaction mixtures containing 2 μl of 80 μM dCTP, dGTP,

Table 1. Sequence-specific oligonucleotide probes used in this study. The probes are listed by *probe number*, the nucleotide sequence (*flanking numbers* indicate first and last nucleotide base positions) and the predicted hybridization patterns with the known *DR4*-associated alleles are also illustrated. The other *DRB1* sequences to which each probe may hybridize are indicated.

DRB1 probes	Nucleotide sequence (5′–3′)							Predicted reactivity patterns of *DR4 DRB1* alleles *0401	*0402	*0403	*0404	*0405	*0406	*0407	*0408	Other DRB1 allele ractivity
400	85	AGA TAC TTC TAT CAC					99	+	+	+	+	+	+	+	+	None
437	103	GAG GAG TCC GTG CGC					117	–	–	–	–	–	+	–	–	*0101-3, 1501-2, 1601-2
457.1	166	CCT GAT GCC GAG TAC					180	+	+	+	+	–	+	+	+	*0101-2, 0301-2, 0802, 1301-2, 1402, 1501-2, 1601-2, PEV
457.2	163	CGG CCT AGC GCC GAG					177	–	–	–	–	+	–	–	–	*0801, 0803, 1303
470.1	209	AGA GGC GGG CCG CGG TGG					226	–	–	–	+	+	–	–	+	*0101-2, 1402
470.2	197	ACA TCC TGG AAG ACG AGC					214	–	+	–	–	–	–	–	–	*0103, 1102, 1301-2
470.3	205	GAG CAG AAG CGG GCC					219	+	–	–	–	–	–	–	–	None
470.4	209	AGA GGC GGG CCG AGG TGG					226	–	–	+	–	–	+	+	–	*0901, 1401, DR6b.2
486.1	250	GGG GTT GTG GAG AGC					264	–	+	+	+	–	+	–	–	*0301, 1102-3, 1301, 1401, 1501
486.2	250	GGG GTT GGT GAG AGC					264	+	–	–	–	+	–	+	+	*0101, 0103, 0302, 0701, 0801-3, 0901, 1001, 1302-3, 1402, 1502, 1601-2, PEV

Table 2. Summary of allele-specific amplification. **Panel A**. Results of DNA amplified from *DR4*, *DRw10*, *DR1*, *DR7*, and *DR9* haplotypes with primer pair EWP4.2/CRX37 at variable annealing temperatures (A.T.). + denotes presence of 280 bp fragment on gel; – denotes absence of fragment. **Panel B**. Results of SSOP hybridization to PCR reaction products. Where there was hybridization of oligonucleotide probes to amplified DNA, probe numbers are listed. *DR4* controls appropriately hybridized to SSOP panel (data not shown); – denotes no hybridization of SSOP to DNA. SSOP #457.1 and #486.1 non-specifically hybridized to *DRw10* and *DR1*-amplified DNA.

A.

	DR				
A.T.(°C)	4	w10	1	7	9
50	+	+	+	+	–
55	+	+	–	–	–
56	+	–	–	–	–
57	+	–	–	–	–
58	+	–	–	–	–
59	+	–	–	–	–
60	+	–	–	–	–
61	+	–	–	–	–
65	–	–	–	–	–

B.

	DR			
A.T.(°C)	w10	1	7	9
50	457.1 486.1	437 457.1 470.1 486.1	–	–
55	486.1	–	–	–
56	–	–	–	–
57	–	–	–	–
58	–	–	–	–
59	–	–	–	–
60	–	–	–	–
61	–	–	–	–

dTTP, 50 mM NaCl, 10% DMSO and either 0.08 μM ddATP, 8 μM ddCTP, 8 μM ddGTP, or 8 μM ddTTP. Reaction mixtures were incubated at 37 °C for 2 min folowed by the addition of 2 μl of 0.25 mM dATP, dCTP, dGTP, dTTP, 10% DMSO. The reaction was allowed to proceed for an additional 2 min before stopping the reaction on ice. Reactions were then boiled for 5 min, placed on ice, and electrophoresed on an 8% polyacrylamide/urea sequencing gel.

Results

Allele-specific amplification. The optimal annealing temperature at which the primer pair EWP4.2/CRX37 could selectively amplify *DR4* alleles was determined with a panel of 13 DNA samples representing four haplotypes tested at nine different annealing temperatures between 50 °–65 °C. Those haplotypes included *DRw10, DR9, DR1*, and *DR7* which differ from *DR4* between nucleotide positions 13–36 by 2, 3, 4, and 5 bases, respectively. As identified by a 280 base pair (bp) fragment on gel electrophoresis, *DR4*-positive haplotypes could be amplified at all annealing temperatures tested except 65 °C (Table 2a). *DRw10* haplotypes could be amplified only at annealing temperatures of 50 °C–55 °C. *DR1* and *DR7* haplotypes were amplified only at 50 °C. *DR9* haplotypes could not be amplified at any of the annealing temperatures tested. Negative and positive reaction mixtures were blotted and hybridized to the panel of SSOP. Only those reaction mixtures which demonstrated the 280 bp amplification product showed detectable hybridization with members of the SSOP panel. As expected, DNA amplified from *DRw10* and *DR1* haplotypes hybridized with certain of the probes in the SSOP panel sharing homologous sequences (Table 2b). Based on these results,

an annealing temperature of 60 °C was selected for *DR4*-specific amplification.

A panel of 100 selected DNA samples (60 *DR4*-negative and 40 *DR4*-positive) representing *DR1*–w18 haplotypes was tested in blinded fashion. Amplification was verified from all 40 *DR4*-positive samples and from none of the 60 *DR4*-negative samples (data not shown). Furthermore, when the 60 *DR4*-negative reaction mixtures were blotted onto membranes and hybridized with the probe panel, there was no detectable hybridization (data not shown). Taken together, these results emphasize the critical importance of temperature in the annealing between template DNA and the *DR4*-specific amplification primer. *DR4*-specific amplification can be achieved using the EWP 4.2/CRX37 primer pair and a 94 °C denaturation, 60 °C annealing, 72 °C extension profile. There was at least a 4 °C error tolerance in annealing temperature, and only at temperatures of 55 °C and lower was amplification of *DR4*-negative haplotypes observed.

Sequence-specific oligonucleotide probe hybridization patterns. Expected hybridization patterns of the SSOP panel with *DRB1*0401*–0408 alleles are illustrated in Table 1. With this panel of ten SSOP, each known *DR4* allele is predicted to yield a unique hybridization pattern. Hybridization of amplified DNA to SSOP #400 is included to confirm the presence of *DR4*. The probes in this panel were designed to hybridize either with consensus sequences (SSOP#457.1, 470.1, 486.1) or with sequences representing *DR4* subtype variation (#437, 457.2, 470.2, 470.3, 470.4, 486.2). Thus the identification of any given *DR4* allele is made by both positive and negative hybridization (Table 1).

Correlation of genotyping by SSOP to cellular typing. Seventy-eight of the 104 individuals tested by SSOP were also phenotyped for HLA-D by using HTC and/or proliferative T cell clones. HLA-D specificities were assigned by cellular typing in 55 (71%) of the 78 individuals tested. In those individuals in whom Dw4, Dw10, Dw15, and DwKT2 were identified, there was a complete ($r=1.00$) correlation with the *DRB1* genotype identified by SSOP. Individuals who phenotyped as Dw13 or Dw14 were found to have one of two distinct alleles. Dw13-positive individuals subtyped with SSOP as either *DRB1*0403* or *0407*, and Dw14-positive individuals subtyped as either *DRB1*0404* or *0408*.

Of the 23 *DR4* individuals for whom an HTC-defined HLA-D specificity could not be assigned, a *DR4 DRB1* allele was identified in each case by SSOP. Two individuals assigned *DR4*, D''blank'' typed as *DRB1*0401*, one as *DRB1*0403*, seven as *DRB1*0405*, two (related individuals) as *DRB1*0406*, three as *DRB1*0407*, and four as *DRB1*0408*. Two of the individuals were *DR4*-homozygous and typed by SSOP as *DRB1*0405/0407* and *DRB1*0404/0408*. Amplified DNA from two of the 23 D''blank'' individuals showed novel hybridization patterns that did not correspond with known *DR4* alleles (see below). These results demonstrate that SSOP can identify sequence polymorphisms within the first domain of *DRB1*, even when HLA-D phenotyping by HTC or T cell clones is not informative.

Two novel DR4 DRB1 alleles. DNA from two unrelated Hispanic individuals ''CB'' and ''EC'' each had novel hybridization patterns with the panel of probes. DNA from ''CB'', a *DR4/DRw6*-positive individual with the phenotype HLA-A3,2; B7,39; DR4,w6; DRw52,w53; DQw1,w7, hybridized with SSOP #400, 457.2, 470.1 and 486.1 (Fig. 1). This pattern differed from that of *DRB1*0404* only by the lack of hybridization with SSOP #457.1 (consensus) and by the hybridization with SSOP #457.2. When compared to *DRB1*0405*, the pattern of *DRB1*04.CB* differed only by the hybridization with SSOP #486.1 (consensus) and the lack of hybridization with SSOP #486.2. From these results, it was inferred that the *DRB1*04.CB* gene sequence differed from *DRB1*0405* at nucleotide positions 256–258 (GTG instead of GGT). These results predicted that *DRB1*04.CB* had valine instead of glycine at codon 86. DNA from ''EC'', an individual having the phenotype HLA-A2, w68; Bw61,14; DR4,3; DRw52,w53; DQw4, w2, hybridized with SSOP #400, 457.2, 470.4, and 486.1 (Figure 1). The SSOP pattern predicted that *DRB1*04.EC* had AGC encoding serine at codon 57, and GAG encoding glutamic acid at codon 74.

DNA was isolated from ''CB'' and ''EC'' for selective *DRB1* second exon amplification and direct sequencing. The nucleotide sequences for *DRB1*04.CB* and

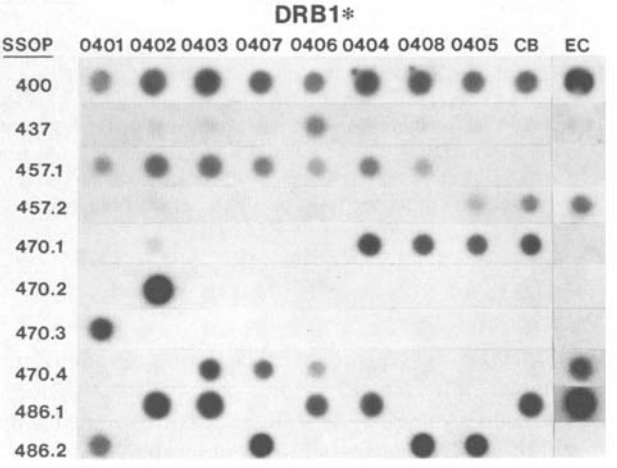

Fig. 1. Dot blot hybridization of *DR4 DRB1* alleles. Shown is an actual hybridization of *DR4* SSOP with amplified DNA from *DRB1*0401–0408*, **04.CB* and **04.EC* genes.

DRB1*04.CB

```
         50        60        70        80        90       100       110       120
          *         *         *         *         *         *         *         *
      CATTTCTTCAACGGGACGGAGCGGGTGCGGTTCCTGGACAGATACTTCTATCACCAAGAGGAGTACGTGCGCTTC
       H  F  F  N  G  T  E  R  V  R  F  L  D  R  Y  F  Y  H  Q  E  E  Y  V  R  F

       130       140       150       160       170       180       190       200
         *         *         *         *         *         *         *         *
GACAGCGACGTGGGGGAGTACCGGGCGGTGACGGAGCTGGGGCGGCCTAGCGCCGAGTACTGGAACAGCCAGAAGGACCTC
 D  S  D  V  G  E  Y  R  A  V  T  E  L  G  R  P  S  A  E  Y  W  N  S  Q  K  D  L

      210       220       230       240       250       260       270
        *         *         *         *         *         *         *
CTGGAGCAGAGGCGGGCCGCGGTGGACACCTACTGCAGACACAACTACGGGGTTGTGGAGAGCTTCACA
 L  E  Q  R  R  A  A  V  D  T  Y  C  R  H  N  Y  G  V  V  E  S  F  T
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DRB1*04.EC

```
         50        60        70        80        90       100       110       120
          *         *         *         *         *         *         *         *
      CATTTCTTCAACGGGACGGAGCGGGTGCGGTTCCTGGACAGATACTTCTATCACCAAGAGGAGTACGTGCGCTTC
       H  F  F  N  G  T  E  R  V  R  F  L  D  R  Y  F  Y  H  Q  E  E  Y  V  R  F

       130       140       150       160       170       180       190       200
         *         *         *         *         *         *         *         *
GACAGCGACGTGGGGGAGTACCGGGCGGTGACGGAGCTGGGGCGGCCTAGCGCCGAGTACTGGAACAGCCAGAAGGACCTC
 D  S  D  V  G  E  Y  R  A  V  T  E  L  G  R  P  S  A  E  Y  W  N  S  Q  K  D  L

      210       220       230       240       250       260       270
        *         *         *         *         *         *         *
CTGGAGCAGAGGCGGGCCGAGGTGGACACCTACTGCAGACACAACTACGGGGTTGTGGAGAGCTTCACA
 L  E  Q  R  R  A  E  V  D  T  Y  C  R  H  N  Y  G  V  V  E  S  F  T
```

Fig. 2. DRB1 second exon sequences for *DRB1*04.CB* and *DRB1*04.EC.*

```
                    HVR I               HVR II                                  HVR III

DR4     HLA-D     6   10        20        30        40        50        60        70        80        90
DRB1    SPECI-    .   .         .         .         .         .         .         .         .         .
ALLELE  FICITY    RFLE--KSECHFFNGTERVRFLDRYFHNQEEYVRFDSDVGEYRAVTELGRPDAEYWNSQKDLLEQRRAAVDTYCRHNYGVVESFT

*0401   Dw4       ----QV-H------------------YH-------------------------------------K--------------G----
*0402   Dw10      ----QV-H------------------YH---------------------------------I--DE-------------------
*0403   Dw13.1    ----QV-H------------------YH----------------------------------------E----------------
*0407   Dw13.2    ----QV-H------------------YH----------------------------------------E-----------G----
*0406   DwKT2     ----QV-H------------------YH---S------------------------------------E----------------
*0404   Dw14.1    ----QV-H------------------YH---------------------------------------------------------
*0408   Dw14.2    ----QV-H------------------YH----------------------------------------------------G----
*0405   Dw15.1    ----QV-H------------------YH-----------------------S----------------------------G----
*04.CB   nd                 ----------------YH-----------------------S---------------------------------
*04.EC   nd                 ----------------YH-----------------------S----------------E----------------
```

Fig. 3. Deduced *DRB1* first domain amino acid sequences for *DR4*-associated alleles. A (–) indicates identity with the consensus sequence. References for the previously published sequences are: *DRB1*0401* (Gregersen et al. 1986); *DRB1*0402* (Gregersen et al. 1986); *DRB1*0403* (Cairns et al. 1985); *DRB1*0404* (Gregersen et al. 1986; Cairns et al. 1985); *DRB1*0405* (Gregersen et al. 1986); *DRB1*0406* (Gregersen et al. 1987); *DRB1*0407* (Gao et al. 1990; Lanchbury et al. 1990; Land et al. 1990); and *DRB1*0408* (Gregersen et al. 1989; Gao et al. 1990; Lanchbury et al. 1990). *DRB1*04.CB* and **04.EC* are new *DR4*-associated alleles. nd indicates that no HLA-D specificity has been defined and no appropriate HTC is available.

*DRB1*04.EC* are illustrated in Figure 2 and the deduced amino acid sequences for the *DR4* family of alleles including *DRB1*04.CB* and *DRB1*04.EC* are shown in Figure 3. As predicted by oligonucleotide typing, the *DRB1* second exon sequence for *DRB1*04.CB* was identical to that of *DRB1*0405* except for GTG at nucleotide positions 256–258 encoding valine. Together, *DRB1*0405* and *DRB1*04.CB* represent the third example of two *DR4* alleles that differ from each other only at codon 86. The other such pairs are *DRB1*0403* and *0407,* and *DRB1*0404* and *0408.* The *DRB1*04.EC* gene sequence is identical to *DRB1*04.CB* except at nucleotide positions 220–222 where GAG encodes glutamic acid instead of GCG encoding alanine. From nucleotide positions 172 to 270, the sequence of the *DRB1*04.EC* gene is identical to either *DRB1*0403* or *DRB1*0406.* From

Table 3. Expected hybridization patterns for *DR4*-homozygous, *DRB1*-heterozygous individuals expressing different pairs of *DR4* alleles. Alleles abbreviated as follows: 1, *DRB1*0401*; *DRB1*0402*; 3, *DRB1*0403*; 4, *DRB1*0404*; 5, *DRB1*0405*; 6, *DRB1*0406*; 7, *DRB1*0407*; 8, *DRB1*0408*; CB and EC (new *DR4* alleles).

DRB1 probes	Unique patterns 1 2	1 3	1 4	1 5	1 6	1 7	1 8	1 CB	1 EC	2 3	2 4	2 5	2 6	2 7	2 8	2 CB	2 EC	3 4	3 7	3 EC	4 6	4 8	4 CB	5 6	5 7	5 8	5 CB	5 EC	6 7	6 8	6 CB	6 EC	7 8	7 EC	CB EC	3 5	7 CB	8 EC	3 8	4 7	4 5	8 CB	3 6	6 6	3 CB	4 EC
400	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
437	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+	−	−
457.1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
457.2	−	−	−	+	−	−	−	+	+	−	−	+	−	−	−	+	+	−	−	+	−	−	+	+	+	+	+	+	−	−	+	+	−	+	+	+	+	+	−	−	+	+	−	−	+	+
470.1	−	−	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−	+	−	−	+	+	+	+	+	+	+	+	−	+	+	−	+	−	+	+	+	+	+	+	+	+	−	−	+	+
470.2	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
470.3	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
470.4	−	+	−	−	+	+	−	−	+	+	−	−	+	+	−	−	+	+	+	+	+	−	−	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+
486.1	+	+	+	−	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
486.2	+	+	+	+	+	+	+	+	+	−	−	+	−	+	+	−	−	−	+	−	−	+	−	+	+	+	+	+	+	+	−	−	+	+	−	+	+	+	+	+	+	+	−	−	−	−

nucleotide positions 211 to 270, *DRB1*04.EC* is also identical to two non-*DR4* alleles, *DRB1*1401* and *DR6b.2* (Gorski et al. 1990). All four alleles have GAG encoding glutamic acid at codon 74. Therefore, *DRB1*04.EC* appears to be a novel allele having arisen either by point mutation or recombination. Point mutation would have involved adenine ↔ cytosine at nucleotide position 221. Recombination between two *DR4* parental alleles may be postulated to have occured between nucleotide positions 172–219, whereas recombination between *DR4* and either *DRw14* or *DR6b.2* parental alleles may be postulated to have occured between nucleotide positions 211–219.

Genotyping of DR4-homozygous, DRB1-heterozygous samples. The hybridization patterns expected for the 42 possible *DR4*-homozygous, *DRB1*-heterozygous combinations are illustrated in Table 3. Ten *DR4*-homozygous, *DRB1*-heterozygous combinations do not have unique patterns. With the addition of an oligonucleotide probe constructed to the TAC (consensus) sequence at codon 37, *DRB1*0403/0406* could be readily distinguished from *DRB1*0406/0406*. SSOP panels used by other investigators (Gao et al. 1990; Lanchbury et al. 1990) do not include probes constructed to nucleotide positions 103–117 (SSOP #437) and 166–180 (SSOP #457.1). In our experience, the inclusion of probes constructed to the TCC sequence at codon 37 and the GAT sequence at codon 57 is crucial to the genotyping of *DR4*-homozygous, *DRB1*-heterozygous samples. Without SSOP #437,20 combinations do not have unique patterns; without the use of SSOP #457.1,10 additional combinations cannot be distinguished, and SSOP panels which exclude both SSOP #437 and #457.1 cannot distinguish 23 additional combinations (data not shown). One of our goals in designing a molecular genotyping method for the *DR4* family of alleles was to enable accurate genotyping of *DR4*-homozygous, *DRB1*-heterozygous samples. The full panel of 10 SSOP represents the most informative approach which satisfies this goal.

Our panel of 104 *DR4*-positive individuals included 13 who were presumed to be *DR4*-homozygous by serologic typing. Two of the 13 *DR4*-homozygous individuals were found to be homozygous for *DRB1*0401*. Eleven of the 13 were found to have two distinct *DR4* alleles: *DRB1*0401/0402* (*N=1*); *DRB1*0404/0408* (*N=1*); *DRB1*0401/0408* (*N=4*); *DRB1*0402/0404* (*N=1*); *DRB1*0405/0407* (*N=1*); *DRB1*0401/0407* (*N=1*); and *DRB1*0401/0405* (*N=1*). Amplified DNA from one of the 11 *DR4*-homozygous, *DRB1*-heterozygous individuals hybridized to SSOP #400, 457.1, 470.1, 470.4, 486.1 and 486.2, and therefore genotyped as either *DRB1*0404/0407* or *DRB1*0403/0408*. An informative family study defined the genotypes as *DRB1*0404/0407*. These results demonstrate that *DR4*-homozygous, *DRB1*-heterozygous individuals can

Table 4. Relative distribution of *DRB1* alleles among *DR4* haplotypes.

DRB1 Allele	Associated HLA-D specificity	Relative frequency (%)
**0401*	Dw4	49
**0402*	Dw10	5
**0403*	Dw13.1	2
**0407*	Dw13.2	12
**0406*	DwKT2	1
**0404*	Dw14.1	10
**0408*	Dw14.2	11
**0405*	Dw15	8
**04.CB*	nd	1
**04.EC*	nd	1
		100%

Study population included 95 *DR4*-positive unrelated individuals representing 102 different *DR4* haplotypes. nd, not defined.

be accurately genotyped in most cases. In those instances where *DRB1*-heterozygous combinations do not have unique patterns (Table 3), a definitive assignment can only be made by an informative family study.

Relative DR4 allele frequencies and novel DR4 DRB1-DQ associations. We calculated the distribution of *DR4* alleles in our study population (Table 4). *DRB1*0401* (Dw4) was the most common allele (49%) observed among the 102 different *DR4* haplotypes, and *DRB1*0407* (Dw13.2) and *0408* (Dw14.2) were the next most common (12% and 11% respectively). Also noteworthy was the identification of *DRB1*0406* (DwKT2) in a Caucasian family. This allele has been previously identified only in the Oriental population in association with *DQw3*. The *DRB1*0406*-bearing Caucasian haplotype we identified was associated with *DQw4*. Eight unrelated Caucasians (8%) typed as *DRB1*0405* (Dw15.1). Like *DRB1*0406, DRB1*0405* was originally described as occurring only in Oriental populations. Our findings confirm a recent report by other investigators (Lanchbury et al. 1990) that this *DR4* allele can also occur in non-Oriental populations.

Discussion

We have used SSOP hybridization with amplified DNA and a panel of ten oligonucleotide probes to study variation within the *HLA-DR4* family of alleles among 117 different *DR4* haplotypes. In most cases, individuals heterozygous for two *DR4* alleles could be precisely genotyped by the methods we describe. The presence of two new *DRB1* sequence polymorphisms was signalled by novel patterns of hybridization which provided rapid identification and characterization of previuosly undetected and undefined alleles. Overall, we observed ten distinct *DR4*-associated alleles in the material analyzed. These results, along with those described by other investigators (Gao et al. 1990; Lanchbury et al. 1990), indicate the precision with which *DR4 DRB1* typing can be performed by SSOP, especially in those circumstances where conventional typing methods are not fully informative.

In our study, and in the previous studies of *DRB1* diversity, primers were designed so that only *DR4-DRB1* sequences could be amplified. This was possible because *DR4* alleles have a unique polymorphism in the first HVR of *DRB1*. This sequence, specific for all known *DR4* alleles (5′-CCACGTTTCTTGGAGCAGGTTAAA-3′), is located between nucleotide positions 13 to 36. On the other hand, the oligonucleotide probes designed to identify the individual *DR4* alleles can hybridize to other non-*DR4 DRB1* alleles (Table 1). For this reason, errors could occur if DNA from non-*DR4* alleles were amplified inadvertently. When applied in a two phase system that allows selective amplification of a specific family of alleles, SSOP can be informative even in *DR4*-homozygous, *DRB1*-heterozygous individuals who have two genes simultaneously amplified by the primer pair.

In the course of our studies we discovered two new *DR4* subtypes. The subtype provisionally designated *DRB1*04.CB* is closely related to the *DRB1*0405* (Dw15) allele but can be distinguished by the presence of a GTG sequence encoding valine instead of GGT encoding glycine at codon 86 in the *DRB1* first domain. A valine → glycine substitution at codon 86 occurs in two other *DR4* allelic variants, Dw13 (*DRB1*0403 and 0407*) and Dw14 (*DRB1*0404* and *0408*), and also in two non-*DR4* allelic pairs *DRw15* (*DRB1*1501* and *1502*) and *DRw13* (*DRB1*1301* and *1302*). The phenomenon of dimorphism at codon 86 in the first domain of the DRB1 α-helix with the interchange of hydrophobic valine and glycine residues suggests that this site plays a significant role in peptide binding and/or T cell receptor interactions. Based on the three-dimensional structure of class I molecules (Bjorkman et al. 1987) as extrapolated to class II molecules (Brown et al. 1988), codon 86 of the DRβ chain is located at the carboxy terminus of the α-helix. Conservative (glycine/valine) amino acid changes at codons 85 and 86 of the DRβ chain can be recognized by T cells (Flomenberg 1989; Eckels et al. 1990; Zeliszewski et al. 1990). The *DRB1*04.EC* subtype may have arisen from recombination between other *DR4* alleles, namely *DRB1*0405* or *DRB1*04.CB* and *DRB1*0403* or *0406*. The generation of novel alleles as a result of point mutation and/or recombination has been observed in other *DRB1* families. Such so called gene conversion events are believed to represent the mechanism by which *DRB1*0301* (Gorski and Mach 1986), *DRB1*1102* (Bell et al. 1987), *DRB1*1303* (Tiercy et al. 1990; Petersdorf et al. 1990) and *DRB1 "PEV"* (Petersdorf et al. 1990) were generated.

In our analysis of *DR4* haplotypes, we also observed some novel DR-DQ associations. The *DRB1*0406* (DwKT2) allele (8W407) was first described in an HLA-homozygous typing cell (A2, B51, DR4, DRw53, DQw3) derived from a Japanese donor. The *DRB1*0406* allele identified in a Caucasian family in our study showed an unusual *HLA-DQw4* association. In most populations, *DR4* has been linked with various subtypes of *DQw3* (30–50% *DQw7* and 50–70% *DQw8*; Lee et al. 1990; Todd et al. 1987; Stephens et al. 1989), although exceptions to these common associations have been described (Awad et al. 1990).

Because of the extensive sharing of individual sequence polymorphisms among different *DRB1* alleles, unambiguous typing by SSOP hybridization depends partly on the specificity of the initial amplification step. Although amplification parameters must be precisely determined for each individual primer pair, conditions that allow SSOP to function in an "allele-specific" manner can be defined. An allele-specific approach has the advantage of providing unambiguous results in nearly all cases, even among individuals in whom both *DRB1* alleles are amplified simultaneously by the primer pair. In contrast, nonspecific amplification of all *DRB1* alleles will always yield two amplification products from heterozygous individuals. Although this alternative approach has the advantage of not requiring prior knowledge of the *DR* type, there remains the disadvantage that certain hybridization patterns will have ambiguous interpretations as was seen in some individuals even with allele-specific amplification. In such cases, the ambiguity would have to be deduced either by subsequent allele-specific amplification or by an informative family study.

In summary, the *HLA-DR4* family is comprised of at least ten distinct alleles characterized by sequence polymorphism within the *DRB1* first domain. Utilization of the panel of oligonucleotide probes developed in this study enables the rapid genotyping of *DR4*-homozygous, *DRB1*-heterozygous samples and permits the clear identification of previously unrecognized *DR4* variants. This system thus represents the most effective and efficient approach available for typing the *DR4* family of alleles.

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