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Diversity associated with the second expressed *HLA-DRB* locus in the human population

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Abstract Although diversity within the *HLA-DRB* region is predominantly focused in the *DRB1* gene, the second expressed *DRB* loci, *DRB3*, *DRB4*, and *DRB5*, also exhibit variation. Within *DRB1*15* or *DRB1*16* haplotypes, four new variants were identified: 1) two new *DRB5* alleles, *DRB5*0104* and *DRB5*0204*, 2) a haplotype carrying a *DRB1*15* or **16* allele without the usual accompanying *DRB5* allele, and 3) a haplotype carrying a *DRB5*0101* allele without a *DRB1*15* or **16* allele. The evolutionary origins of these haplotypes were postulated based on their associations with the *DRB6* pseudogene. Within *HLA* haplotypes which carry *DRB3*, a new *DRB3*0205* allele and one unusual *DRB3* association were identified. Finally, two new null *DRB4* alleles are described: *DRB4*0201N*, which exhibits a deletion in the second exon, and a second allele, *DRB4*null*, which lacks the second exon completely. Gene conversion-like events and variation in the number of functional genes through reciprocal recombination and inactivation contribute to the diversity observed in the second expressed *HLA-DRB* loci.

Introduction

The *DR* subregion of the human major histocompatibility complex (*MHC*) contains a single *DRA* locus and multiple *DRB* loci. The product of the *DRA* gene and of a functional *DRB* gene associate to form the DR molecule, the predominant class II molecule present on the surface of cells of the immune system. Either one or two expressed *DRB* loci are found within this subregion in different *HLA* haplotypes (Bodmer et al. 1992). To date, all human *MHC* appear to contain a *DRB1* locus, the most centromeric of the *DRB* loci. This locus specifies the DRβ chain of DR molecules such as DR1, DR15, DR16, and DR3. There is an association between particular *DRB1* alleles and the presence of a specific expressed second locus. In *HLA* haplotypes carrying alleles *DRB1*15* or **16*, the second locus is designated *DRB5* and encodes the DR51 molecules originally identified using serologic typing. In haplotypes carrying alleles *DRB1*03*, **11*, **12*, **13*, and **14*, the second locus is designated *DRB3* and encodes DR52 molecules. In haplotypes carrying alleles *DRB1*04*, **07*, and **09*, the second locus is designated *DRB4* and encodes DR53 molecules. Finally, in haplotypes carrying *DRB1*01*, **08*, and **10* alleles, a second expressed *DRB* locus is usually not present. Known exceptions occur within the *DRB1*01* and **15* haplotypes. Rare *DRB1*01* haplotypes do carry an expressed *DRB5* locus while, conversely, *DRB1*15* haplotypes without a *DRB5* allele have been described [("LUM") (Wade et al. 1993; Bidwell et al. 1992; Gebuhrer et al. 1992)]. Another exception is the *DRB1*07* haplotype with a *DRB4* allele which is not expressed (Sutton et al. 1989).

Other *HLA-DRB* loci present in the *DR* subregion, including *DRB2*, *DRB6*, *DRB7*, *DRB8*, and *DRB9*, are pseudogenes and are not expressed as protein products. The defects in these loci range from deletions of entire exons to alterations in the regulatory regions that flank the exons. As with the functional *DRB* loci, not all nonexpressed loci are present in a haplotype. For example, the *DRB6* locus is found associated with haplotypes carrying *DRB1*01*, **10*, **15*, and **16* alleles (Corell et al. 1992; Figueroa et al. 1991); *DRB7* and *DRB8* with haplotypes carrying *DRB1*04*, **07*, and **09* alleles (Day et al. 1994; Larhammar et al. 1985); and *DRB2* with haplotypes carrying a *DRB3* locus (Vincek et al. 1992; Rollini et al. 1987).

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Table 1 Cell lines utilized in the study[a]

Cell line	Ethnic origin[b]	*DRB1*[c]	*DRB3/4/5*	*DRB2/6/7/8*	*DQA1*	*DQB1*	GenBank accession No.
GN045	AFA	**1503/*1303*	***5*0104***/*3*0202*	*6*0201/2**	**0102/*0201*	**0602/*0202*	U31770
GN00151	EC	**1601/*1101*	***5*0204***/*3*0202*	ND[d]	**0102/*05013*	**0502/*0301*	U59685
GN049	AFA	**1503/*0408*	*4*0103*	*6*0101/7*0101/8**	**0102/*0302*	**0602/*0301*	
GN053	AFA	**1503/*1101*	*3*0202*	*6*0101/2**	**0102/*05013*	**0602/*0301*	
GN098	AFA	**1602/*0701*	*4*01011*	*6*0101/7*0101/8**	**0102/*0201*	**0502/*0202*	
GN001	EC	**0101/*1301*	*5*0101/3*0101*	*6*0201/2**	**01MV/*0103*	**0501/*0603*	
GN027	EC	**0701*	***5*0101***/*4*01011*	*6*0201/7*0101/8**	**0102/*0201*	**0602/*0202*	
GN087	EC	**0301*	***5*0101***/*3*0201*	*6*0201/2**	**0102/*05011*	**0602/*0201*	
RXT	EC	**0301*	***5*0101***/*3*0101*	*6*0201/2**	**0102/*05011*	**0602/*0201*	
GN068	EC	**1401/*0701*	***3*0205***/*4*01011*	*2*/7*0101/8**	**01MV*[e]*/*0201*	**05031/*0202*	U36826
GN080	AFA	**0806*	***3*0301***	negative	**0102*	**0602/*0501*	
GN016	EC	**0701/*1602*	***4*0201N***/*5*02*	*6*0202/7*0101/8**	**0201/*0102*	**0202/*0502*	U50061, U70543, U70544, U70545
GN017	EC/NA	**0701/*1401*	***4*null***/*3*0201*	*2*/7*0101/8**	**0201/*01MV*	**0202/*05031*	U70542
GN020	EC	**0701/*1501*	***4*null***/*5*0101*	*6*0201/7*0101/8**	**0201/*0102*	**0202/*0602*	U70542
GN076	EC/NA	**0701/*0101*	***4*null***	*6*0101/*0101/8**	**0201/*01MV*	**0202/*0501*	U70542
GN092	EC	**0701/*1501*	***4*null***/*5*0101*	*6*0201/7*0101/8**	**0201/*0102*	**0202/*0602*	U70542
TTH-18	EC	**0701/*1302*	***4*null***/*3*0301*	*2*/7*0101/8**	**0201/*0102*	**0202/*0609*	U70542

[a] Cell pellets are available to interested investigators
[b] EC, European Caucasion; AFA, African American; NA, native American
[c] All *DRB*, *DQA1*, and *DQB1* alleles identified by SSOP. Some alleles were also identified by sequence analysis
[d] ND, not determined
[e] MV, *01/04/05*

While the diversity of the human *DRB1* locus is extensive with over 160 alleles currently defined, the diversity at the second expressed *HLA-DR* locus is more restricted (Bodmer et al. 1995). Fewer than ten alleles have been described for each locus, *DRB3*, *DRB4*, and *DRB5*. The nonexpressed *HLA* loci may also be polymorphic; for example, three alleles have been described at the *DRB6* locus (Corell et al. 1992). To explore the diversity of the second expressed *DRB* locus, cells carrying unusual alleles at these loci identified by polymerase chain reaction-sequence specific oligonucleotide probe (PCR-SSOP) typing were selected and the variant second locus identified by sequence specific priming (SSP) and/or DNA sequencing. The *DRB* pseudogenes carried by these cells were also identified.

Materials and methods

Human cells utilized in the study are described in Table 1. Reference cells carrying well-defined *HLA-DRB* alleles were obtained from the International Histocompatibility Workshop (IHWS) panels (Yang et al.1989)(12th IHWS, S. Marsh, unpublished data). DNA and RNA extraction, cDNA synthesis, PCR amplification, and SSOP typing were performed according to the procedures described previously (Robbins et al. 1995; Shaffer et al. 1992; Kimura et al. 1992). Allele-specific amplification was carried out in PCR buffer (Invitrogen, San Diego, CA) containing 60 mM Tris-HCl, pH 8.5, 25 mM $(NH_4)_2SO_4$, 100 nM each primer, 100 μM each dNTP, 1.25 units AmpliTaq (Perkin Elmer, Foster City, CA) and 0.5 μg genomic DNA in a total volume of 50 μl in a Perkin Elmer 2400 thermal cycler. HotWax Beads (Invitrogen) incorporating Mg were used as a hot start. The $MgC1_2$ concentration varied for different primer combinations. [Primer sequences, combinations, and amplification conditions can be obtained from the corresponding author.] In some cases, amplification primers contained M13

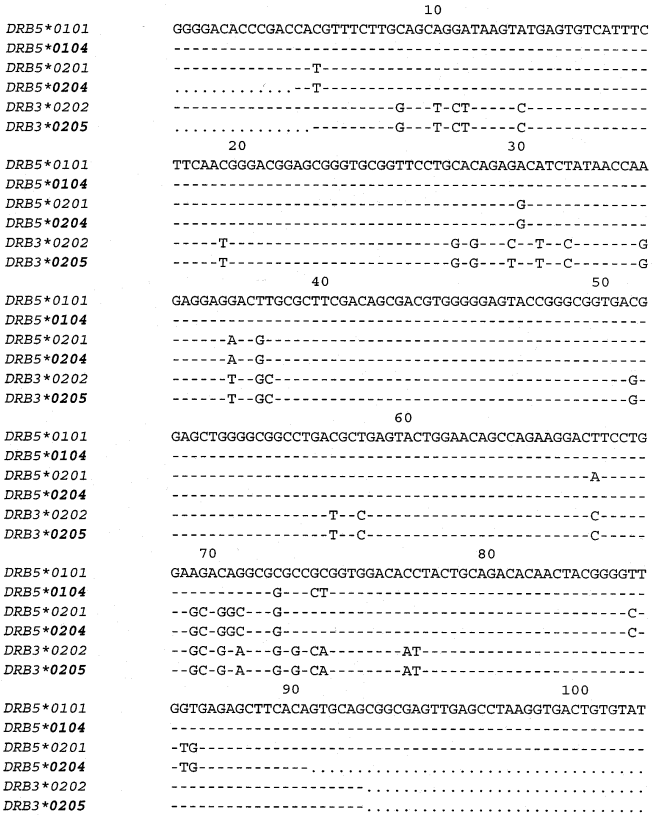

Fig. 1 Comparison of nucleotide sequences of new *DRB5* alleles and *DRB3* allele with other related alleles (Marsh and Bodmer 1995)

Table 2 SSP-PCR profiles of DR2 and DRB5 cells

Primer pairs	GN049	GN053	GN098	GN001	GN027	GN087	RXT	9295[a]	9293[a]	Primer pair specificity
DRBAMP-2[b], DRBR/91[c, d]	+	+	+	–	–	–	–	+	+	DRB1*15/16
DRBAMP-B4D[b], DRBR/7011[c, e]	+	+	–	–	–	–	–	+	+	DRB1*15, DRB5*02
DRBAMP-B4D, DRBR/7013-4[c, e]	–	–	+	+	+	+	+	+	+	DRB1*16, DRB5*01
DRBAMP-B5[b], DRBR/91[d]	–	–	–	+	+	+	+	+	+	DRB5*
3XDRB/101[c], 3XDRBR/133[c, f]	+	+	+	–	–	–	–	+	+	DRB1*15/16
3XDRB5/105[c], 3XDRBR/179[c, f]	–	–	–	+	+	+	+	+	+	DRB5*
DRBAMP-1[b], DRBAMP-B[b, f]	–	–	–	+	–	–	–	–	–	DRB1*01

[a] IHWS
[b] Kimura and co-workers (1992) or 12th IHWS, M. Fernandez-Vina and J. D. Bignon, unpublished data
[c] DRBR/91, GCTCACCTCGCCGCTGCA; DRBR/7011, CCGCGCCTGCTCCAGGAT; DRBR/7013-4, TAGGTGTCCACCGCGGCG; 3XDRB/101, TCCAACCTAAGGTGACTGTA; 3XDRBR/133, CTTCTCTTCCTGGCCGTTCA; 3XDRB5/105, GGTGACTGTATATCCTGCAAG; 3XDRBR/179, GTGAGAGGGCT(T/C)GTCACG
[d] Primer pair anneals within exon 2
[e] Primer pair anneals in intron 1 and exon 2
[f] Primer pair anneals in exon 3

Table 3 Distribution of *DRB5null* alleles

Population	*DRB1*15/16+* Tested	*DRB5null* (%)	*DRB1*-associated with null (No.)[a]	*DRB6*-associated with null
European Caucasian	1670	1 (0.06)	**15011* (1)	**0101*
Asian	106	0		
Hispanic/Native American	270	0		
African American	303	15 (5.3)	**15011* (2) **1503* (13)	**0101* **0101*

[a] *DRB1*1602* was observed in GN098 but not found in the survey of additional individuals

(-21M13 forward primer: 5′TGTAAAACGACGGCCAGT and M13 reverse primer: 5′CAGGAAACAGCTATGACC) sequences at the 5′ end to allow Dye Primer Cycle Sequencing. The reaction mixtures were heated at 98 °C for 30 s followed by four cycles: denaturation at 98 °C for 10 s, annealing at 5 °C above the primer pair optimal temperature for 30 s, and extension at 72 °C for 60 s. The next 32 cycles were: denaturation at 96 °C for 20 s, annealing at the primer pair optimal temperature for 30 s, and extension at 72 °C for 60 s. To monitor the specificity of the amplification primer pairs, DNA containing alleles which should or should not amplify with the primer pairs were included as controls in the development of the PCR conditions.

PCR products (4 µl) were run on a 1% agarose gel and visualized with ethidium bromide. The remaining reaction (45 µl) diluted with 450 µl water was loaded onto a MicroconTM-100 cartridge (Amicon, Beverly, MA) to remove unincorporated dNTP and primers and to reduce the final volume to 40 µl. These templates were utilized for DNA sequence analysis.

All sequencing reagents, including the Ready Reaction Dye Terminator and Dye Primer Cycle Sequencing Kits, were purchased from Perkin Elmer. The manufacturer's protocol was followed for sequencing reactions and the product was analyzed on Applied Biosystems 373 or 377 Automated DNA Sequencers (Foster City, CA). One to 5 µl of purified PCR product were used for each reaction. A minimum of two PCR reactions were performed for each primer pair and the sequences in both orientations were obtained (Robbins et al. 1995; McGinnis et al. 1995). Allele designations were assigned by the WHO Nomenclature Committee in July 1995 (*DRB5*0104*), November 1995 (*DRB3*0205*), April 1996 (*DRB4*0201N*), and June 1996 (*DRB5*0204*).

DRB probes to identify *DRB6* alleles included DRB8001 (*DRB6*0101*, exon 2); DRB8003 (*DRB6*0201*, exon 2); DRB8004 (*DRB6*0202*, exon 2; Kimura et al. 1992) and to identify *DRB7* included: DRB7020 W (*DRB7*0101*, exon 2; 12th IHWS, M. Fernandez-Vina and J.D. Bignon, unpublished data). Probes to identify *DRB4* included: DRE301GN (*DRB4*01011*, exon 3, 5′CGGAACGGCCAGGAAGAC) and DRE303GN (*DRB4*0103*, exon 3, 5′CGGAACAGCCAGCAACAG). Other probes to identify *DRB*, *DQB1*, and *DQA1* have been described elsewhere (Kimura et al. 1992; Shaffer et al. 1992; Wade et al. 1993; Hurley et al. 1995).

Results

New DRB5 alleles confound generic DRB typing

DNA from cell GN045 (Table 1) was amplified with *DRB*-specific PCR primers, DRBAMP-A and DRBAMP-B (Kimura et al. 1992), and probed with a series of oligonucleotide probes. Based on the hybridization pattern, the oligotype was assigned as *DRB1*1313*, *DRB1*1503*, *DRB3*02*, *DRB5*0101*. When group-specific amplification was used to amplify each *DRB* allele for subsequent identification by oligonucleotide probe hybridization, the probe DR7030GN (5′GAAGACAGGCGGGCCCTG) did not hybridize to the amplified *DRB1*13* DNA as expected but hybridized instead to the amplified *DRB*5 DNA.

cDNA was amplified from GN045 using primers which amplify only the *DRB5* allele from the cell. Primer combinations were chosen to generate at least two overlapping fragments for direct sequence analysis of the new allele

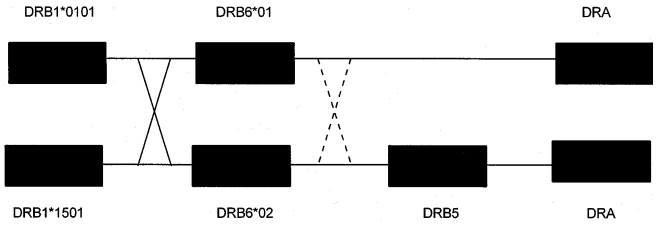

Fig. 2 Organization of the *DRB1*01* and *DRB1*15* haplotypes (Corell et al. 1992; Figueroa et al. 1991). The approximate site of the recombination event generating the *DRB5* deletion observed in GN049, GN053, and GN098 and the *DRB5*-positive *DRB1*01* haplotype in GN001 is indicated. The approximate site of the recombination event generating the LUM haplotype is shown as a *dotted line*

[(Fig. 1)(Robbins et al. 1995)]. The new allele, *DRB5*0104*, differs from the most closely related allele, *DRB5*0101*, only at codons 72–74. This region is shared with *DRB1*08* alleles, suggesting that this allele arose through a gene conversion-like event involving two different *DRB* loci.

DNA from GN151 hybridized to probes in *DRB* and *DRB5*-specific amplifications with a pattern that suggested *DRB5*0201/0202* except for failure to hybridize with probe DRB7011 (Kimura et al. 1992). DNA sequence analysis confirmed that the new allele, *DRB5*0204*, was virtually identical to *DRB5*0201* only differing in codon 67 ATC/Ile (*DRB5*0201*) → TTC/Phe (Fig. 1). Although GN151 is heterozygous (Table 1), the new *DRB5* allele is likely associated with SSOP-defined *DRB1*1601*, *DQA1*0102*, *DQB1*0502*.

Loss of DRB5 in some DR2 haplotypes

Cells GN049, GN053, and GN098, which appeared to carry *DRB1*15* or **16*, were identified by SSOP typing and sequencing but did not appear to carry the associated *DRB5* allele as defined by lack of reactivity with PCR-SSOP and SSP directed to sequences found in both exon 2 and exon 3 (Tables 1, 2). For example, primer pair DRBAMP-B4D and DRBR/7013–4 which should amplify *DRB1*16* and *DRB5*01* was not able to amplify DNA from GN049 and GN053 but could amplify DNA from cells GN098 (*DRB1*16*), GN027, GN087, and RXT (cells described below) and reference cells (IHWS 9293 and 9295). An analysis of 2349 additional individuals typed as *DRB1*15* or **16* for a United States bone marrow registry of volunteer donors in the first six months of 1995 identified one Caucasian and 15 African American individuals as *DRB5null* (Table 3), suggesting that this haplotype is predominantly limited to African Americans.

The *DRB5null* was found in association with *DRB1*1501, *1503,* and **1602*. Since all of the *DRB5null* cells tested in this study carry a *DRB6*0101* allele which is commonly associated with *DRB1*01* and *DRB1*10* in contrast to the usual *DRB1*15/16* association with *DRB6*02* (Corell et al. 1992) and since the *DRB6* locus lies between *DRB1* and *DRB5* loci, it is likely that the

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                Intron-1                                    <- ->EXON-2
DRB4*0101101    gaggccgcccgtgtgtgaccggatcgttcgtgtccccacag CACGTTTCTTG
DRB4*0201N      .......---------------------------------- -----------
                  10                        20
DRB4*0101101    GAGCAGGCTAAGTGTGAGTGTCATTTCCTCAATGGGACGGAGCGAGTGTGGAAC
DRB4*0201N      ----------------------------------------**********
                      30                        40
DRB4*0101101    CTGATCAGATACATCTATAACCAAGAGGAGTACGCGCGCTACAACAGTGACCTG
DRB4*0201N      ------------------------------------------------------
                           50                        60
DRB4*0101101    GGGGAGTACCAGGCGGTGACGGAGCTGGGGCGGCCTGACGCTGAGTACTGGAAC
DRB4*0201N      ------------------------------------------------------
                               70                        80
DRB4*0101101    AGCCAGAAGGACCTCCTGGAGCGGAGGCGGGCCGAGGTGGACACCTACTGCAGA
DRB4*0201N      ------------------------------------------------------
                                     90                ->EXON-3
DRB4*0101101    TACAACTACGGGGTTGTGGAGAGCTTCACAGTGCAGCGG  AAAGACCCAGCCC
DRB4*0201N      ------------------------------.......  -------------
                  110                       120
DRB4*0101101    CTGCAGCACCACAACCTCCTGGTCTGCTCTGTGAATGGTTTCTATCCAGGCAGC
DRB4*0201N      ------------------------------------------------------
                      130                       140
DRB4*0101101    ATTGAAGTCAGGTGGTTCCGGAACAGCCAGGAAGAGAAGGCTGGGGTGGTGTCC
DRB4*0201N      ------------------------------------------------------
                           150                       160
DRB4*0101101    ACAGGCCTGATCCAGAATGGAGACTGGACCTTCCAGACCCTGGTGATGCTGGAA
DRB4*0201N      ------------------------------------------------------
                               170
DRB4*0101101    ACAGTTCCTCGGAGTGGAGAGGTTTACACCTGCCAAGTGGAGCA
DRB4*0201N      -------------------------------------------
```

Fig. 3 Comparison of nucleotide sequence of new *DRB4* allele with *DRB4*0101101* (Marsh and Bodmer 1995)

DRB5null haplotypes arose by recombinations between *DRB1* and *DRB6* genes of *DRB1*15/16* and *DRB1*01* (or **10*) haplotypes (Fig. 2). This hypothesis is supported by the characterization of cells such as GN001 (Table 1) which carry *DRB1*0101* in association with *DRB5*0101* and *DRB6*0201* (also observed by Fischer et al. 1993). These recombination events may be facilitated by the sequence similarity between these closely related haplotypes (Svensson et al. 1996). In contrast, the *DRB5null* cell line LUM carries *DRB1*1501* in association with *DRB6*0201* (Corell et al. 1993), suggesting that the recombination breakpoint for the LUM haplotype occurred between *DRB6* and *DRB5* loci.

Loss of DRB1 in some DR2 haplotypes

Three cells, GN027, GN087, and RXT, which carried the *DRB5*0101* allele yet did not carry the expected associated *DRB1* allele, e.g., *DRB1*15*, *DRB1*16*, or *DRB1*01*, were identified by PCR-SSOP hybridization and SSP (Table 2). These methods were directed to detect exons 2 and 3 and intron 1 of *DRB1*15/16* alleles and exon 2 of *DRB1*01* alleles. For example, primer pair *DRB*AMP-B4D and DRBR/7011 which should amplify all *DRB1*15* and *DRB5*02* alleles, was not able to amplify DNA from these cells; however, the primer pairs did amplify DNA from cells GN049, GN053, and GN001 (described above) and reference cells carrying the appropriate alleles. Based on commonly observed *DR/DQ* associations, it is likely that the *DRB1*null* haplotype carries *DQA1*0102* and *DQB1*0602* alleles (Table 1). These alleles are usually found in association with *DRB1*1501* or *DRB1*1503* alleles (Fig. 2). Thus, it is likely that all or a portion of the *DRB1*15* locus was deleted, leaving the flanking *DQ* and *DRB6*0201* alleles intact.

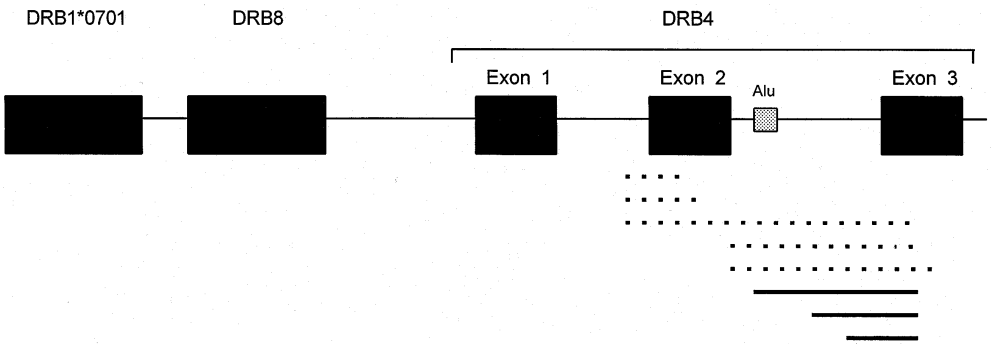

Fig. 4 Exon/intron organization of the *DRB4* locus showing the location of amplification primers used to detect the deletion. Amplification products shown as *dotted lines* were not obtained, suggesting that one or both primers sites have been deleted. Amplification products shown as *solid lines* were obtained; the amplification products located 3′ of the *alu* repeat region were characterized by DNA sequence analysis. No amplification was obtained using cDNA and primer pairs annealing in exon 1 and exon 3 or in exon 2 and exon 3. The pattern of amplification coupled with the presence of the *DRB8* locus suggests that the deletion occurred between *DRB8* and the 5′ boundary of *DRB4* intron 2

*A new DRB3 allele identified in association with DRB1*1401*

DRB3-amplified DNA from GN068 hybridized with probes DRB1011, DRB2809, and DRB3711 (Kimura et al. 1992). The hybridization pattern, specifically the hybridization with probe DRB2809, suggested that a new allele was present. DNA sequence analysis confirmed that the new allele was virtually identical to *DRB3*0202*, only differing from *DRB3*0202* in a single base substitution at codon 30 (CAC → TAC) shared with *DRB3*0301* (Fig. 1). This base substitution results in a change from histidine to tyrosine. Although GN068 is heterozygous (Table 1) and no family studies are available, the new *DRB3* allele is likely associated with SSOP-defined *DRB1*1401*, *DQA1*01*, and *DQB1*05031* alleles. Based on similarity, this allele could have arisen by a gene conversion-like event altering the common *DRB1*1401*, *DRB3*0202* haplotype.

An unusual DRB3 association is observed

Cell GN080 (Table 1) was typed by PCR-SSOP as *DRB1*0806*, *DRB3*0301*. No other *DRB1* or *DRB3/4/5* alleles could be identified by hybridization or in attempts to directly sequence or clone and sequence other alleles. Since *HLA-A*, *-B*, *-DQA1*, and *-DQB1* loci are heterozygous as determined by serology (A30,68;B50,72) and PCR-SSOP typing, either the *DRB3*0301* allele is not found in association with a *DRB1* allele or the *DRB3* allele is in linkage with a *DRB1*0806* allele. *HLA* haplotypes expressing *DRB1*08* do not usually carry a second expressed *DRB* locus. Based on sequence similarity, it has been suggested that *DRB1*08* haplotypes were formed by an unequal crossing-over event which fused a *DRB1*08* first and second exons with 3′ *DRB3* exons (Gorski 1989). One possibility is that GN080 carries the original *DRB1*08* haplotype prior to this recombination event; however, the cell does not carry the *DRB2* locus found associated with *DRB1*08* and *DRB3* in an ancestral haplotype observed a nonhuman primate (Kasahara et al. 1992). A more likely possibility is that this cell carries a haplotype which has lost its *DRB1* (and *DRB2*) allele. In support of this hypothesis, the *DQ* alleles carried by the cell are sometimes found in association with *DRB1*11/*13*. Unfortunately, family members were not available to confirm this observation by segregation analysis.

Two deletions result in null DRB4 alleles

Cells GN016, GN017, GN020, GN076, GN092, and TTH-18 were typed as *DRB1*0701* (Table 1); however, the *DRB4* alleles from these cells gave unexpected SSOP hybridization patterns. A combination of *HLA-DRB* exon 2- specific amplification and SSOP typing indicated that the DNA from GN016 failed to bind to a *DRB4*-specific probe, DRB2810, yet hybridized to another *DRB4*-associated probe, DRB7009 (Kimura et al. 1992). The splicing site polymorphism found in the *DRB4*0101102N* allele (Sutton et al. 1989) was not present as determined by direct sequencing of the PCR product encompassing intron 1 through exons 2 and 3. Further DNA sequence analysis of the *DRB4*-encoded allele identified an 11 base deletion encompassing codons 23–26 of *DRB4* (Fig. 3). This delection results in a frame shift and stop codon, TAA, just 3′ of the deletion site. Thus, it is not likely that a normal *DRB4*-encoded protein product is expressed by this cell. Two distinct but related variants of *DRB4null* transcripts were observed by reverse transcriptase-PCR and direct sequencing. One has the usual exon 1-exon 2 junction with the 11 base pair (bp) deletion at codons 23–26; the other uses a cryptic splice acceptor site in intron 1 (Sutton and Knowles 1990; Hinsdale et al. 1993) with an additional 731 bp insertion between exons 1 and 2 (data not shown). Sequence analysis of exon 3 of the *DRB4* allele and SSOP typing suggests that this allele, *DRB4*0201N*, is most closely related to *DRB4*0101101*.

The combination of *HLA-DRB* exon 2-specific amplification and SSOP typing indicated that PCR product from cells GN017, GN020, GN076, GN092, and TTH-18 failed to bind to *DRB4*-specific probes DRB2810 and DRB7009. Use of the PCR-SSP method to scan exons 1 and 2 and intron 1 failed to detect any product with multiple primer combinations suggesting that this region is missing from these cells (Fig. 4; data not shown). The combination of

Table 4 Association of nonexpressed *DRB* alleles with haplotypes

Haplotype	No. Tested	Nonexpressed alleles
*DRB5*01*-positive (predominantly *DRB1*15*-associated)	346 random, 23 selected	*DRB6*0201* (368[a]) *DRB6*0202* (1)
*DRB5*02*-positive (predominantly *DRB1*16*-associated)	40 random, 7 selected	*DRB6*0202* (45) *DRB6*0201* (2)
*DRB1*0101, DRB5*-negative; *DRB1*1001, DRB5*-negative	266 random, 8 selected	*DRB6*0101* (274)
*DRB1*04* *DRB1*07* *DRB1*09*	640 random, 33 selected	*DRB7*0101* (673)
DRB4-positive	21 selected	*DRB8*-positive (21)
DRB3-positive	11 selected	*DRB2*-positive (10) *DRB2*-negative (1[b])
*DRB1*08*	6 selected which did not carry *DRB1*03/11/12/13/14*	*DRB2*-negative (6)

[a] Number tested
[b] GN080

DRB exon 3- and intron 2-specific amplification, sequencing, and SSOP typing indicated that the *DRB4* exon 3 is present and identical to *DRB4*0101101*. Pseudogenes *DRB7*01* and *DRB8** were detected by SSOP and SSP. These genes are located between *DRB1* and *DRB4* loci, suggesting that the deletion event took place between *DRB8* and *DRB4* loci.

The *DRB1*0701* sequence of exon 2 was obtained from GN016, GN017, GN020, GN076 and GN092. The *DQ* alleles encoded by these haplotypes are likely to be *DQA1*0201, DQB1*0202*.

Nonexpressed DRB alleles are associated with specific haplotypes

Additional typing of a total of 1281 cells selected at random and 87 additional cells selected to carry specific alleles confirmed observations made with the cells described above. Results are presented in Table 4.

Discussion

It is thought that the *DRB* loci arose over evolutionary time by gene duplication and divergence (Klein 1987; Satta et al. 1996). The tight association observed between particular *DRB1* alleles and the second expressed *DRB* locus may be the result of the limited recombination between *DRB1* and the second locus. Thus, for example, duplication and subsequent divergence may have created the *DRB4* locus from the locus encoding *DRB1*04/*07/*09* alleles and this combination has not yet been reshuffled by recombination (Svensson et al. 1996). Another hypothesis is that selection has subsequently maintained this close association. For example, particular common HLA-DR combinations such as DR15/DR51, DR3/DR52 or DR4/DR53 may represent molecules with complementary functional properties in terms of antigen binding repertoire or in the selection of optimal T-cell receptors for antigen recognition. However, while the function of the *DRB1*-encoded molecule in antigen-specific responses has been clearly established, the importance of the second DR molecule in immune responses remains to be completely defined (Rosen-Bronson and Jaraquemada 1991; Wucherpfennig et al. 1994; Kuijpers et al. 1992).

A number of mechanisms appear to be operating to generate diversity in the second expressed *DR* locus. Although the absolute numbers of alleles for the second expressed *HLA-DRB* loci are a fraction of the number of *HLA-DRB1* alleles, it appears that a similar gene conversion-like mechanism has created the diversity observed. Indeed, the new alleles described this report, *DRB5*0104*, *DRB5*0204*, and *DRB3*0205*, differ from previously defined alleles at those loci by polymorphisms previously observed in other *DRB* alleles. The *DRB1*15* and **16* haplotypes appear particularly prone to gene contraction and expansion where haplotypes which have lost either *DRB1* or *DRB5* loci are likely to have arisen by unequal crossing over with closely related haplotypes in the *DRB1*01*, **15*, **16*, or **10* cluster. The loss of loci by inactivation is most evident for the *DRB4* locus and suggests that this second locus may be less important in the immune response and may represent the early stages leading to a pseudogene. The loss of a locus by deletion is one of three mechanisms observed to inactivate alleles. Similar observations have been made in nonhuman primates, where the *DRB* loci appear to be expanding and contracting (Figueroa et al. 1994; Slierendregt et al. 1994) and underscore the dynamic nature of the major histocompatibility complex.

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Note added in proof

*B4*null* was assigned as *DRB4*030IN* by the WHO Nomenclature Committee in December 1996.