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Identification of a polymorphic variant associated with HLA-DQw3 and characterized by specific restriction sites within the DQ β-chain gene

(histocompatibility antigens/class II genes/major-histocompatibility-complex/polymorphisms/type I diabetes)

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ABSTRACT **Restriction endonuclease digestion of genomic DNA from 24 lymphoblastoid cell lines homozygous for the HLA class II specificity DQw3, followed by hybridization with a DQ β-chain cDNA probe, identified a genomic polymorphism with variable *Bam*HI and *Hin*dIII recognition sites. This restriction fragment pattern was found for several haplotypes associated with the DQw3 specificity, including some haplotypes positive for the HLA-DR specificities DR4, DR5, DRw8, and DRw12. The variant fragment pattern corresponds precisely with the reactivity of a monoclonal antibody, A-10-83, previously shown to define a serologic split of DQw3. Serologic detection of the specific DQw3.1 genomic polymorphism indicated that the corresponding DQ β-chain variants are expressed. This polymorphic restriction fragment pattern, then, represents a selective marker for DQ β-chain genes that appear to define a DQ β-chain-associated specificity, here called DQw3.1.**

The *HLA* class II region encodes a series of polymorphic glycoproteins that form cell-surface heterodimers each consisting of one α and one β chain (1–3). These class II molecules are encoded by genes clustered within three loci, *DP*, *DQ*, and *DR* (4–9), and are functionally implicated as regulatory signals in intercellular communication during the immune response (10–12).

The phenotypic hallmark of the *HLA* complex is a high degree of structural and functional polymorphism. Class II polymorphic differences have been associated with immune-response differences in many species and with disease susceptibilities in man (12–14). Detailed analysis of such polymorphisms should aid in understanding the molecular basis for associations between *HLA* and disease.

We have used genomic blotting to investigate polymorphisms associated with *DQβ* class II genes on haplotypes expressing the HLA-DR4 and DQw3 specificities. Previous studies of haplotypes identified by conventional serology as sharing the common allospecificity DR4 have demonstrated structural variability in both DR and DQ β-chain polypeptides (15–17). In addition, several investigators have reported polypeptide variation among DQw3-positive class II molecules isolated from cells that differ in DR phenotype (18–20). Using restriction endonuclease digestion and hybridization with cDNA probes for *DRβ*, *DQβ*, and *DQα* genes, we previously demonstrated considerable polymorphisms at the *DQβ* and *DQα* loci among *DR4,DQw3*-homozygous cell lines (21). We now report the results of hybridization studies designed to investigate specific *DQ*-associated restriction endonuclease fragment length polymorphisms (RFLP) that directly relate to class II functions. The endonucleases *Hin*dIII and *Bam*HI were used to identify a specific *DQβ* genomic polymorphism that directly corresponds to a serologically defined specificity related to HLA-DQw3. This genomic polymorphism subdivides the DQw3 serologic specificity and identifies a particular DQβ variant that has been described as a marker of susceptibility to type I diabetes.

MATERIALS AND METHODS

Homozygous Cell Lines (HCL). Epstein–Barr virus-transformed B-lymphoblastoid cell lines were prepared from *HLA-D*-homozygous donors as described (15). Eighteen DR4-, three DR5-, one DRw12-, two DRw8-, one DRw9-, and one DR7-positive HCL were used (Table 1). Each of these HCL was also HLA-DQw3-positive, except for two (HAS-15 and KT-3) which express an undefined DQ antigen designated HLA-DQw-blank.

Restriction Endonuclease Digestion of Genomic DNA. Cellular DNA was digested for 18 hr at 37°C with ≥2 units of *Bam*HI or *Hin*dIII (Bethesda Research Laboratories) per μg of DNA. Digestion was monitored for completeness by minigel analysis of both the genomic digest and of phage λ DNA to which an aliquot of the genomic digestion mixture had been added. Reactions were stopped by addition of EDTA to 10 mM. DNA digests were concentrated by addition of 0.67 volume of 5 M ammonium acetate and 2 volumes of ethanol, chilling 30 min in a dry ice/methanol bath, and pelleting the DNA by centrifugation at 11,600 × *g* for 8 min. Pellets were resuspended in 10 mM Tris·HCl, pH 7.4/1 mM EDTA.

Southern Blotting. Restriction endonuclease-digested DNA (12 μg per lane) was applied to 0.7% agarose gels in 40 mM Tris acetate, pH 7.4/ 1 mM EDTA. Phage λ DNA digested with *Hin*dIII and phage φX174 DNA digested with *Hae* III (Bethesda Research Laboratories) were included as molecular size markers. Gels were run at 30 volts for 18 hr and then stained with ethidium bromide. The gels were soaked 15 min in 0.25 M HCl, followed by treatment with 0.5 M NaOH/1.5 M NaCl for 1 hr to denature the DNA. Gels were neutralized by soaking 1 hr in 0.5 M Tris·HCl, pH 7.0/3.0 M NaCl, and the DNA was transferred to nitrocellulose (Schleicher & Schuell) by the method of Southern (23). After transfer, the filters were rinsed in 2× standard saline citrate (SSC; 1× = 150 mM NaCl/15 mM sodium citrate, pH 7.0) and then baked 18 hr at 80°C.

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Abbreviations: HCL, homozygous cell line(s); RFLP, restriction fragment length polymorphism(s); kb, kilobase(s).

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Table 1. Genomic and serologic polymorphic variants among HLA-DQw3 cell lines homozygous for *HLA-D*

				Monoclonal antibody reactivity		RFLP		
						*Bam*HI		*Hin*dIII
HCL	HLA-D	HLA-DR	HLA-DQ	17.15*	A-10-83*	12-kb	6.9/3.7-kb	3.4-kb
ER	Dw4	DR4,w53	DQw3	+	+	−	+	+
WALK	Dw4	DR4,w53	DQw3	+	+	+	+	+
NIN	Dw4	DR4,w53	DQw3	+	+	−	+	+
HA	Dw4	DR4,w53	DQw3	+	−	+	−	−
PRIESS	Dw4	DR4,w53	DQw3	+	−	+	−	−
MJ-4	Dw4	DR4,w53	DQw3	+	−	+	−	−
EM	Dw10	DR4,w53	DQw3	+	−	+	−	−
FS	Dw10	DR4,w53	DQw3	+	−	+	−	−
JHa	Dw13	DR4,w53	DQw3	+	+	−	+	+
SST	Dw13	DR4,w53	DQw3	+	−	+	−	−
THO	Dw14	DR4,w53	DQw3	+	−	+	−	−
BIN-40	Dw14	DR4,w53	DQw3	+	−	+	−	−
LS-40	Dw14	DR4,w53	DQw3	+	−	+	−	−
KT-2	LD"KT2"	DR4,w53	DQw3	+	−	+	−	−
KT-13	LD"KT2"	DR4,w53	DQw3	+	−	+	−	−
TAS	LD"TAS"	DR4,w53	DQw3	+	−	+	−	−
SWEIG	Dw5	DR5,w52	DQw3	+	+	−	+	+
JGL	Dw5	DR5,w52	DQw3	+	+	−	+	+
JME	Dw5	DR5,w52	DQw3	+	+	−	+	+
HLF	DB6	DRw12,w52	DQw3	+	+	−	+	+
8854	Dw8	DRw8,w52	DQw3	+	+	−	+	+
LUY	Dw8	DRw8,w52	DQw3	+	+	−	+	+
KOZ	DB5	DRw9,w53	DQw3	+	−	+	−	−
JK	Dw11	DR7,w53	DQw3	+	−	+	−	−

*Ninth International Histocompatibility Workshop numbers (22): 9w790 (A-10-83), 9w969 (17.15).

Hybridization and Washing of Filters. Filters were prehybridized for 6–18 hr at 42°C in 5× SSC/25 mM sodium phosphate, pH 6.5/5× Denhardt's solution (1× is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/5 mM EDTA/salmon testes DNA (250 μg/ml)/50% (vol/vol) formamide/1% (wt/vol) glycine. Probe DNA [the 1080-nucleotide insert pDR-β-1 in plasmid pBR322 (24), generously provided by P. Peterson] was labeled by nick-translation with [α-^{32}P]dCTP (New England Nuclear) to ≈10^8 cpm/μg of DNA. The labeled DNA was denatured by treating 10 min with 0.1 M NaOH and then neutralized with 0.1 M Tris·HCl (pH 8.0) and 0.1 M HCl. The probe was diluted to 2 × 10^6 cpm/ml in 5× SSC/25 mM sodium phosphate, pH 6.5/2× Denhardt's solution/5 mM EDTA/salmon testes DNA (250 μg/ml)/50% formamide/10% (wt/vol) dextran sulfate. Filters were incubated for 48 hr at 42°C and then washed three times at room temperature in 2× SSC/0.1% $NaDodSO_4$ for 5 min and three times at 60°C in 0.1× SSC/0.1% $NaDodSO_4$ for 20 min. Filters then were exposed to Kodak XAR-5 film with an intensifying screen at −70°C for 3–7 days.

Microcytotoxicity Assay. A standard two-stage microcytotoxicity assay (25, 26) was performed in which 70 different alloantisera and monoclonal antibodies reactive to DR4-, DRw53-, or DQw3-associated antigens identified in the Ninth International Histocompatibility Workshop (22) were tested on the panel of 26 HCL. Cytotoxicity was determined by eosin dye uptake of dead cells after complement-mediated lysis.

RESULTS

***Bam*HI and *Hin*dIII RFLP from *HLA-DR4* HCL.** Hybridization of a DQβ cDNA probe to *Bam*HI- or *Hin*dIII-digested cellular DNA from 18 *HLA-DR4* HCL is shown in Fig. 1. The DQβ cDNA probe hybridized to *Bam*HI-digested DNA fragments of approximately 12.0, 10.2, 7.0, 6.9, 5.5, 4.9, 4.1, and 3.7 kilobases (kb) (Fig. 1*A*). Of these fragments, the 12.0-kb, 6.9-kb, and 3.7-kb fragments are variable: With the exception of HCL WALK, cells display either the 12.0-kb band or 6.9/3.7-kb bands, but not both. HCL ER, NIN and JHa display the 6.9/3.7-kb polymorphic fragments and lack the 12.0-kb band; HCL WALK displays all three polymorphic fragments. The same DQβ cDNA probe hybridized to three prominent fragments of approximately 7.3 kb, 3.4 kb, and 3.2 kb in *Hin*dIII-digested DNA; a number of fainter bands were observed as well (Fig. 1*B*). The 7.3-kb and 3.2-kb fragments are present in all HCL tested. In contrast, the 3.4-kb fragment is present only in HCL ER, WALK, NIN, and JHa. There is complete concordance between the observed 3.4-kb *Hin*dIII fragment and the presence of the 6.9/3.7-kb *Bam*HI RFLP. Faint bands at 7.0, 5.5, and 4.9 kb represent cross-hybridization with *DRβ* genomic fragments, as previously reported (21).

***Bam*HI and *Hin*dIII RFLP from DR4-Negative HCL.** A similar situation was seen when DNA samples from DQw3-positive, non-DR4 HCL were analyzed. *Bam*HI-digested genomic DNA samples from three *HLA-DR5* HCL, one *HLA-DRw12* HCL, two *HLA-DRw8* HCL, one *HLA-DRw9* HCL, and one *HLA-DR7* HCL (all positive for HLA-DQw3) were hybridized with the same DQβ cDNA probe (Fig. 2*A*). Blots display either a 12.0-kb fragment or 6.9/3.7-kb fragments, as well as a number of fainter bands that differ from comparable *Bam*HI RFLP seen in the *HLA-DR4* HCL. Hybridization of the DQβ cDNA probe to *Hin*dIII-digested DNA from these non-*HLA-DR4* HCL is shown in Fig. 2*B*. Again, these cells display prominent bands at 7.3 kb, 3.4 kb, and 3.2 kb, as well as a number of fainter bands. The 3.4-kb fragment is present in cells SWEIG, JGL, JME, HLF (Herluf), 8854, and LUY. In addition, there is complete concordance between the observed 3.4-kb *Hin*dIII fragment and the presence of 6.9/3.7-kb *Bam*HI RFLP.

A-10-83 (Anti-TA10) Monoclonal Antibody Reactivity Correlates with the Presence of the 6.9/3.7-kb *Bam*HI and 3.4-kb

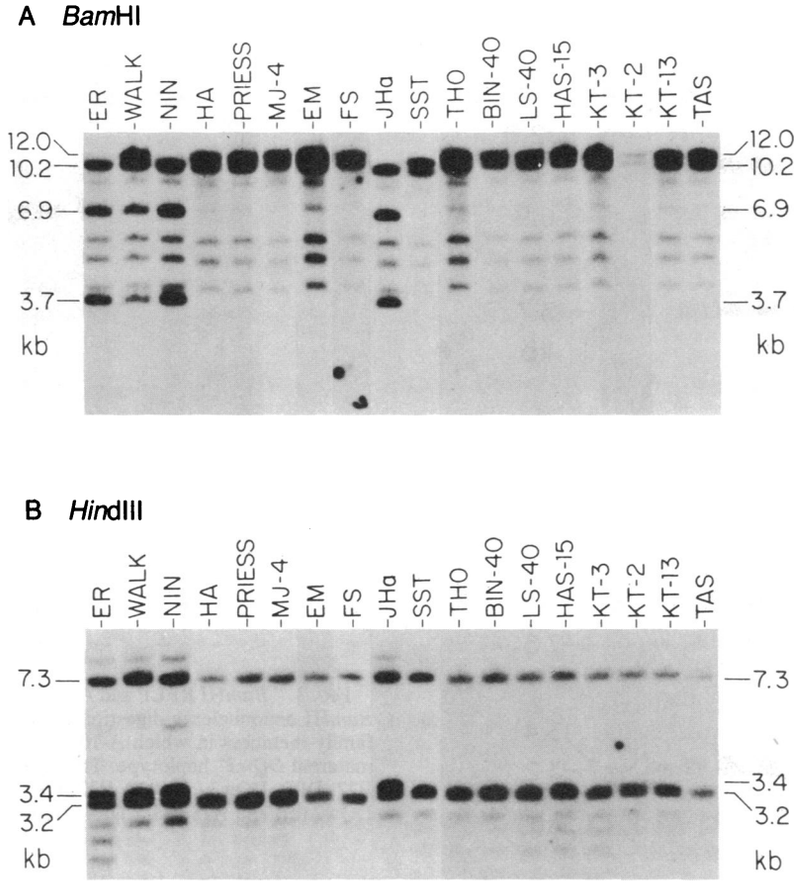

FIG. 1. Southern blot analysis of *Bam*HI-digested (*A*) and *Hin*dIII-digested (*B*) genomic DNA from 18 *HLA-DR4* HCL, hybridized with a DQβ cDNA probe. HCL designations are given above the lanes.

***Hin*dIII RFLP.** We analyzed each of the HCL in this study for reactivity with 70 different alloantisera and monoclonal antibodies directed against DR4-associated, DRw53-associated, and DQw3-associated specificities. From this analysis, a single monoclonal antibody was identified whose pattern of reactivity with the HCL panel correlated precisely with the presence of the 6.9/3.7-kb *Bam*HI and 3.4-kb *Hin*dIII RFLP (Table 1). This antibody, A-10-83, identifies the antigen TA10, which is known to define a serological split of DQw3 and to represent a product of *DQβ* genes, based on two-dimensional gel analysis and limited expression on unstimulated monocytes (22, 27). Among the 24 DQw3-positive HCL tested in the present study, A-10-83 reactivity correlates 100% with the presence of the 6.9/3.7-kb *Bam*HI and 3.4-kb *Hin*dIII RFLP (Table 1). Although several other monoclonal antibodies tested at the Ninth International Histocompatibility Workshop were also identified as TA10-reactive (22), only monoclonal antibody A-10-83 correlated exactly with this RFLP pattern.

Table 1 summarizes the informative monoclonal reactivity and RFLP patterns found. HLA-DQw3-positive cells are all reactive with monoclonal antibody 17.15, a DQw3-specific reagent (22, 28). Those which are A-10-83-reactive all carry the variant *Bam*HI and *Hin*dIII recognition sites, which we designate as the DQw3.1 variant. Cells lacking the specific RFLP are nonreactive with A-10-83; 17.15-positive (DQw3-positive) cells lacking this DQw3.1 marker we designate as DQw3.2.

Segregation of the 6.9/3.7-kb *Bam*HI RFLP with HLA-DQw3.1. To test the linkage of the A-10-83 reactivity pattern with the specific variant RFLP, we analyzed cells derived from family HAR. As illustrated in Fig. 3, the heterozygous maternal phenotype is positive for DQw3.1 (17.15^+,A-10-83^+) and the heterozygous paternal phenotype is positive for DQw3.2 (17.15^+,A-10-83^-). As predicted, the maternal genotype contains the variant 6.9- and 3.7-*Bam*HI bands. Among the three offspring, both A-10-83 reactivity and the *Bam*HI variant bands segregate with the maternal *d* (*DQw3.1*) haplotype. Additional RFLP are apparent in this figure; of note are prominent bands at 6.0 and 2.8 kb that segregate with the paternal *b* (*DQw1, DRw10*) haplotype.

DISCUSSION

We have identified specific genomic polymorphisms, reflected in variable restriction endonuclease recognition sequences, that are coordinately linked to an expressed *HLA* class II polymorphism, detected by monoclonal antibody reactivity. These variant DNA sequences, recognized by at least two different restriction endonucleases, define a *DQβ* allelic polymorphism corresponding to a serologic split of the public specificity HLA-DQw3.

The serologic specificity HLA-DQw3 is present on *HLA-DR4* homozygous cell lines, except for those with the Dw15 specificity, and also on several haplotypes expressing DR5 and some expressing DR7, w8, w9, and w12 (Table 1). The gene for DQw3 has been mapped to the *HLA-DQ* locus by a comparison of the amino acid sequence of purified DQw3 polypeptides with the nucleotide sequences of *DQα* and *β* genes (11). Obviously, DQw3-positive cells are phenotypically heterogeneous, since the DQw3 specificity is expressed by cells that differ in *HLA-D* and *-DR*. In a previous study of DR4-positive HCL, *DQα* and *β* genes associated with DQw3 were found to be quite heterogeneous, as evidenced by a

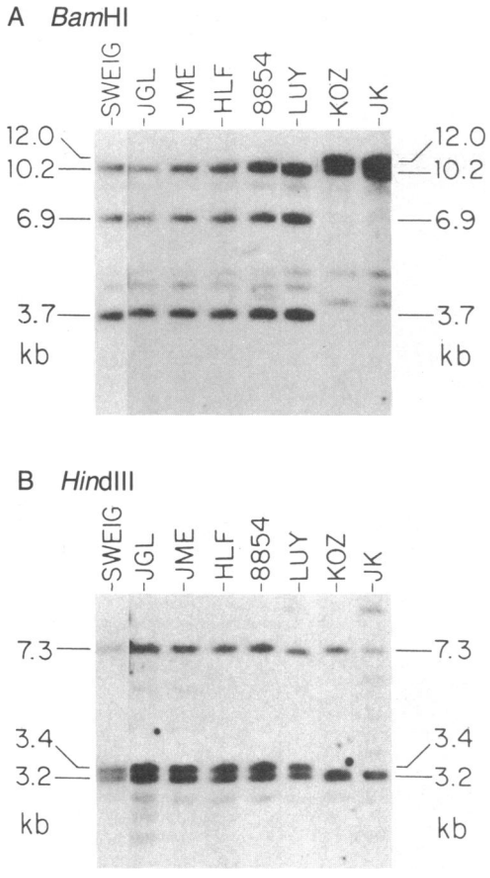

FIG. 2. Southern blot analysis of *Bam*HI-digested (*A*) and *Hin*dIII-digested (*B*) genomic DNA from eight non-*DR4* HCL positive for HLA-DQw3, hybridized with a DQβ cDNA probe.

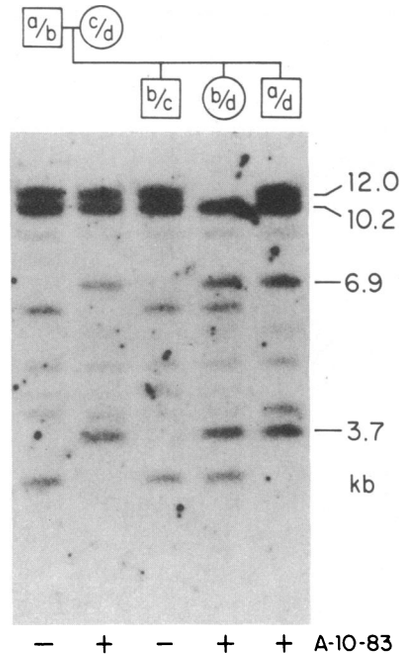

FIG. 3. *Bam*HI RFLP and A-10-83 seroreactivity in family HAR. *Bam*HI endonuclease digestion of genomic DNA is shown for five family members in which A-10-83 seroreactivity segregates with a maternal *DQw3*$^+$ haplotype. The HLA phenotypes shown are A11, B27, DR9, DQw3 (a); A28, B37, DR10, DQw1 (b); A11, B44, DR7, DQw2 (c); and A28, B35, DR4, DQw3(3.1) (d).

number of polymorphic restriction sites that varied among the cells tested (21). We have now directly compared the presence of *DQβ*-associated RFLP among different haplotypes that express the DQw3 specificity and found specific *Hin*dIII and *Bam*HI RFLP that are also reflected in a specific serologic polymorphism.

When *Bam*HI- or *Hin*dIII-digested DNA is hybridized with a DQβ cDNA probe, a 3.4-kb *Hin*dIII band occurs in complete concordance with the presence of 6.9/3.7-kb *Bam*HI RFLP. These two RFLP appear to represent alternative alleles to a 12-kb *Bam*HI fragment and a 3.2-kb *Hin*dIII band associated with *DQβ*. Intrafamily segregation analysis of *Bam*HI-digested DNA confirmed this allelism. HCL WALK displays both of these alleles and therefore appears to be heterozygous for *DQβ*. Among the 24 DQw3-positive HCL tested, monoclonal antibody A-10-83 reactivity correlates 100% with the presence of this polymorphism. Southern blots of DNA digested with a variety of restriction enzymes and probed with *DQα*- and *DRβ*-specific probes did not reveal patterns correlating with this serologic reactivity (data not shown). We consider it very likely, therefore, that the A-10-83 reactivity defines a *DQβ*-associated specificity that represents a split of DQw3 which we call DQw3.1 (A-10-83$^+$).

Although this nucleotide variability corresponds to an expressed molecule with distinct serologic specificity, the *DQ* differences between cells that are DQw3.1 and DQw3.2 are apparently not sufficient to generate a significant proliferative response in mixed-lymphocyte culture. This is evidenced by the observation (Fig. 1 and Table 1) that *HLA-Dw4* HCL may be either DQw3.1 or DQw3.2. Similarly, HLA-Dw13 HCL also may express either DQw3.1 or DQw3.2. This finding is consistent with previous observations that the major stimulating determinant in bulk mixed-lymphocyte reactions is often the product of *DR* genes, and not of *DQ* genes (17). Nevertheless, it is likely that cloned alloreactive T cells may be able to recognize subtle *DQ*-associated variations (E. Mickelson, personal communication).

Previous attempts (21, 30–32) to correlate RFLP on Southern blots with specific HLA serologic typing polymorphisms have led to the general conclusion that, although patterns of shared RFLP cluster within cells of similar typing specificities, consistent nucleotide polymorphisms are not generally reflected in specific alloantigenic polymorphisms. Nevertheless, we now have shown that it is possible to identify specific RFLP that correlate with a precise serologic polymorphism, the TA10-associated marker recognized by monoclonal antibody A-10-83.

The RFLP pattern characterizing the DQw3.1 specificity was not previously recognized as a discrete haplotype marker. In a previous report (33) of DNA polymorphisms associated with insulin-dependent diabetes mellitus (IDDM), DNA samples from patients typed as DR4 or DR3/4 lacked a 3.7-kb *Bam*HI fragment when hybridized with the same *DQβ* probe we used (see Figs. 1 and 2). Our analysis indicates that the absence of this band is not a specific disease marker but rather a *DQβ* polymorphism marking DQw3.2-positive haplotypes. The expression of the DQw3.1 specificity and its associated *DQβ* genotypic variant is indeed markedly decreased in *DR4*-associated IDDM, as well as in unaffected *HLA*-matched family members (unpublished observations). We infer from these data that variability of specific nucleotides (which may generate specific RFLP) represents very precise allelic polymorphisms that are likely to subdivide the broader alloantigenic polymorphisms recognized by conventional HLA typing. We anticipate the use of such nucleotide markers will generate more specific haplotypic markers for analysis of *HLA*-associated function and disease susceptibility.

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