HLA Gene Amplification and Hybridization Analysis of Polymorphism

HLA Matching for Bone Marrow Transplantation of a Patient with HLA-deficient Severe Combined Immunodeficiency Syndrome

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Abstract

The treatment of choice for certain immunodeficiency syndromes and hematological disorders is bone marrow transplantation (BMT). The success of BMT is influenced by the degree of HLA compatibility between recipient and donor. However, aberrant expression of HLA sometimes makes it difficult, if not impossible, to determine the patient's HLA type by standard serological and cellular techniques. We describe here the application of new molecular biological techniques to perform high resolution HLA typing independent of HLA expression. A patient with HLA-deficient severe combined deficiency was HLA typed using in vitro amplification of the HLA genes and sequence-specific oligonucleotide probe hybridization (SSOPH). Two major advances provided by this technology are: detection of HLA polymorphism at the level of single amino acid differences; and elimination of a requirement for HLA expression. Although the patient's lymphocytes lacked class II HLA proteins, polymorphism associated with DR7,w53;DQw2;DRw11a (a split of DR5), w52b (a split of DRw52); DQw7 were identified. The patient's class I expression was partially defective, and typing was accomplished by a combination of serological (HLA-A and -C) and SSOPH analysis (HLA-B). Complete patient haplotypes were predicted after typing of family members [A2;B35(w6);Cw4; DRw11a(w52b);DQw7 and A2;B13(w4);Cw6;DR7(w53); DQw2]. Potential unrelated donors were typed and a donor was selected for BMT.

Introduction

Successful bone marrow transplantation (BMT)[1] is dependent upon the degree of HLA matching between donor/recipient pairs (1). This results from the physiological role of HLA in self-restriction of cellular interactions during an immune response (2). If polymorphic residues in the HLA proteins are mismatched, the immune system may recognize the cells bearing the mismatched HLA as foreign. The consequences of such mismatching include graft-versus-host disease (GVHD), rejection of grafts, and failure to reconstitute a competent immune system (3). These problems are minimized by selection of HLA-matched siblings as donors. Unfortunately, this option is available for only ~ 30–40% of patients who could benefit from a bone marrow transplant. In the remaining patients (60–70%), HLA typing with high resolving power is necessary for selection of an optimally matched, unrelated donor (1, 3).

Traditionally, HLA typing has been accomplished by serological or cellular techniques, which require the presence of detectable levels of HLA proteins on the surface of lymphocytes. In some cases (e.g., HLA-deficient SCID or cellular depletion due to chemotherapy), the levels of the HLA proteins or number of available cells are inadequate to achieve reliable HLA typing. Another limitation of traditional typing methods is the inability to resolve all functionally important HLA alleles. These circumstances have prompted the development of methods for analysis of HLA polymorphism at the genetic level (4, 5).

We describe here the first example of the application of two recently developed techniques, gene amplification and oligonucleotide hybridization, to determine the HLA type of a Caucasian patient with HLA-deficient severe combined immunodeficiency (SCID). This disorder is characterized by combined immunodeficiency associated with defective expression of class I and/or class II HLA products on mononuclear cells. Early reports have described this disorder as bare-lymphocyte syndrome (6). However, recent reports have used the term "HLA-deficient SCID" to acknowledge the presence of non-HLA proteins on the surface of mononuclear cells. Without correction of the disorder, the immunodeficient patient will succumb to overwhelming infection, usually during the first few years of life (6). Although BMT is the treatment of choice, donor selection is impeded by the inability to use standard serological methods to determine the patient's HLA type. We resolved this problem by instituting new molecular biological techniques for HLA typing. HLA genes were selectively amplified (7) from DNA isolated from the patient, family members, and prospective unrelated donors. Polymorphic residues in the amplified DNA were identified by sequence-specific oligonucleotide probe hybridization (SSOPH). SSOPH can discriminate single base pair mismatches (5), which is equivalent to detection of a single amino acid polymorphism in the HLA proteins. This case illustrates the utility of SSOPH in circumstances in which expression of HLA proteins is reduced and/or fine resolution of alleles is required.

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1. *Abbreviations used in this paper:* BMT, bone marrow transplant; GVHD, graft-vs.-host disease; LCL, lymphoblastoid cell lines; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SCID, severe combined immunodeficiency; SSOPH, sequence-specific oligonucleotide probe hybridization.

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Methods

Oligonucleotides. Oligonucleotides were synthesized by a Gene Assembler (Pharmacia Fine Chemicals, Piscataway, NJ) using phosphoramidite chemistry. Oligonucleotides were purified using OPC cartridges (Applied Biosystems, Foster City, CA) or gel electrophoresis.

Cell lines. Lymphoblastoid cell lines (LCL) used here were distributed and characterized by the 10th International Histocompatibility Workshop.

Serological methods. Heparin-treated venous blood was incubated with carbonyl iron, and peripheral blood lymphocytes (PBL) were obtained by centrifugation over Ficoll-Hypaque gradients (1.077 g/ml). Cells were washed, T and B cells were separated by nylon adherence, and HLA typing was performed by standard microlymphocytotoxicity assays (8, 9). Serological specificities were assigned according to WHO nomenclature adopted following the 10th International Histocompatibility Workshop (10).

DNA isolation and amplification. DNA was isolated from blood cells as described previously (11). Primer-directed enzymatic amplification of DNA was accomplished using the polymerase chain reaction as described previously (7). Briefly, 100-μl reaction mixtures contained 1.0–3.0 μg genomic DNA; 200 μM each deoxynucleoside 5′-triphosphate; 1 μM each primer; 50 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$; and 0.01% (wt/vol) gelatin. Primers for amplification of segments of the HLA genes are described in Table I. Samples were boiled for 1 min, transferred to a 94°C heat block, and 1-2 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) were added. The reactions consisted of 30–50 cycles of denaturation (94°C), annealing for 1–2 min (35°C for HLA-DQ; 55°C for HLA-B and -DR), and polymerization (72–74°C) in a programmable heat block (Perkin-Elmer Cetus Instruments). The total time required for the PCR reaction is 3–5 h and theoretically results in a billion-fold amplification. The products of the reaction were characterized by agarose gel electrophoresis of samples (7–20 μl) followed by detection of amplified DNA with ethidium bromide staining.

Hybridization of oligonucleotides. Aliquots of the reaction mixture were denatured by incubation in 0.4 M NaOH, 0.6 M NaCl for five min; neutralized by addition of two volumes of 1 M Tris-HCl, pH 7.0; and applied to Genescreen Plus Membranes (New England Nuclear, Boston, MA) using a slot blot apparatus (Schleicher and Schuell, Keene, NH). Membranes were baked at 80°C for 15 min. ^{32}P-labeling of oligonucleotides (12), hybridization, washing of membranes, and autoradiography were performed as described previously (13), except for the following minor modifications. Filters were prehybridized in 5× Denhardt's (1× Denhardt's is 0.2 mg/ml each albumin, polyvinylpyrrolidone, and Ficoll), 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 10 mM Na phosphate, and 5 mM EDTA for 1–3 h at 68°C. Filters were hybridized overnight in 5× SSC, 10× Denhardt's solution, 20 mM Na phosphate, 100 μg/ml herring DNA, 5 mM EDTA, 7% SDS, and 0.5–1.1 × 10^6 cpm/ml oligonucleotide probe. Total processing time from receipt of samples to development of autoradiograms can be 2 d.

*Table I. Primers for Amplification of First Domain Exon**

Name	Sequence
DR BETA, (16–23)	5′-ATTTCTTCAATGGGACGGAGC
DR BETA (87–94)	5′-CGCCGCTGCACTGTGAAGCTCTC
DQ BETA, (14–20)	5′-TGTGCTACTTCACCAACGGG
DQ BETA (83–89)	5′-CGTGCGGAGCTCCAACTGGT
Class I (1–6)	5′-GCTCCCACTCCATGAGG
Class I (85–91)	5′-CGGCCTCGCTCTGGTTG

* The names of the primers include numbers that correspond to the location of the amino acid residues that are encoded by each primer.

*Table II. Oligonucleotide Probes**

Probe name	Probe sequence	Reference for sequence of allele
DR Locus		
E 58	GCCTGATGAGGAGTACTGG	14, 15, 16
L 30	CTGGAAAGACTCTTCTATA	17
D 70.1	CTGGAAGACAGGCGGGCCG	14
H 30	CTGGAGAGACAC	18
F 37	GGAGTTCGTGCG	Gorski, unpublished
DE 70	CTGGAAGACGAGCGGGCCG	18
FL 37	GGAGTTCCTGCG	18
A 70	CTGGAGCAGGCGCGGGCCG	12, 19
H 33	TACTTCTATCACCAAGAGG	20
N 77	GGACAACTACTG	18
ErCV 28	CTGGAAAGATGCATCTATA	21
N1I26	GGAACCTGATCA	20
dsdvge 41‡	GACAGCGACGTGGGGGAG	
DQ Locus		
E 45	GGAGGTGTACCG	22
EF 46	GGGGGAGTTCCG	23
iynre 31	CTATAACCGAGA	
B Locus		
A 46	CGAGGATGGCGCCCCGGGC	24
egpeyw 55	GGAGGGGCCGGAGTATTGG	

* Probe sequences were designed by identifying allele-specific and consensus regions in previously published sequences for HLA-DR, -DQ, and B loci.

‡ This probe does not hybridize with the nonpolymorphic B4 locus.

Selection and nomenclature of oligonucleotide probes. Sequences used for generating oligonucleotide probes were derived from comparison of published and unpublished data. A sequence conserved in all alleles was used to prepare a control probe. The sequences of all known alleles were aligned and regions were chosen that could identify individual alleles. Some alleles do not contain a unique sequence and must be identified with a combination of probes that detect sequences present in two or more alleles. The oligonucleotide probes used in the case presented here are listed in Table II along with the references from which the total sequence data were originally reported. The oligonucleotide probes are named according to the corresponding amino acid sequences using single letter symbols. Consensus probes are named in lower case letters. Thus, the DR-β consensus sequence is named "dsdvge." Polymorphic probes are designated by upper case letters symbolizing the residue(s) that differ(s) from the consensus sequence followed by a number indicating the position of the first polymorphic residue in the name of the probe. For example, the probe "E 58" encodes the consensus sequence with the exception of the glutamic acid (E) codon at position 58. The polymorphic probes can be associated with one or a few HLA-DR alleles as indicated by comparison of nucleic acid sequences. When a single serological specificity is associated with multiple nucleic acid sequences, the alleles are given the serological designation followed by a lower case letter (e.g., DRw11a and DRw11b).

Results

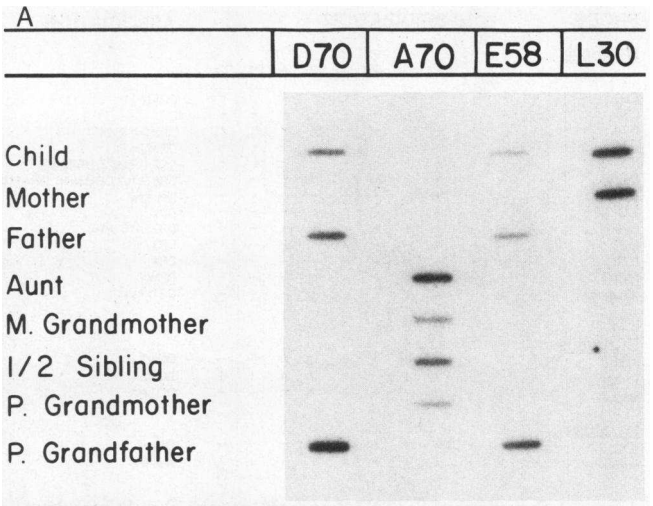
The clinical aspects of the patient's disease will be reported elsewhere (Casper, J. T., M. J. Chusid, and P. A. Kirchner,

manuscript in preparation). Briefly, the diagnosis of HLA-deficient SCID was based on the infant's immunodeficiency manifested by a progressive pneumonia due to *Pneumocystis carinii,* absence of serum immunoglobulins and lack of expression of HLA antigens on the mononuclear cells. The HLA deficiency was indicated by the lack of HLA on the cell surface (by FACS analysis using anti-β_2 microglobulin and anti-DR monoclonal antibodies) and failure to detect class I or class II antigens by serological typing methods. Initially, HLA typing of the patient samples was completely negative except for weak reactions with two of six HLA-A2 antisera. After culturing cells with a variety of mitogens and lymphokines, HLA-C could be detected, and the HLA-A typing improved. Products of the HLA-B, -DR, and -DQ loci were not detectable after any procedures.

The HLA-DR, -DQ, and -B types of the patient were determined by SSOPH of amplified DNA. Polymorphic regions of the HLA genes were amplified from genomic DNA using the primers described in Table I. Electrophoretic analysis of the products of each amplification revealed a single ethidium bromide staining band of predicted size (data not shown). Amplified DNA, from case samples and a control panel, was analyzed by SSOPH with the probes listed in Table II.

Oligonucleotide probes were designed to detect polymorphisms that are associated with HLA phenotypes that would be expected in the patient, based on serological analysis of samples from family members. The relevant HLA-DR types detected in family members were HLA-DR2,4(w53),7(w53), and w11(w52). A preliminary SSOPH for DR2,4,7,w11 and w52 indicated that the patient was DR7,w11,w52. The HLA-DR region of the major histocompatibility complex of DR7 and DRw11 individuals usually contains two loci encoding DRβ chains. The DR7 and DRw11 products are encoded by the B1 locus, and the supertypic specificities DRw52 and DRw53 are encoded by the B_3 and B_4 loci, respectively. There are three different B_1 locus sequences that can give rise to an HLA-DRw11 serotype and three different B_3 sequences that are associated with the DRw52 specificity. A second level of SSOPH provided discrimination between the three possible types of HLA-DRw11 sequences and three possible DRw52 sequences. The resulting hybridization pattern is consistent with the presence of sequences derived from cell lines that were typed as DRw11a and DRw52b. SSOPH was also utilized to confirm the presence of anticipated alleles of HLA-DRw53, HLA-B (B13), and HLA-DQ (DQw2 and DQw7).

An example of primary SSOPH data is shown in Fig. 1. The patient (child) row shows positive hybridization with three polymorphic probes, D70, E58, and L30. Positive hybridization of the E58 probe is consistent with the presence of a DRw11 allele. The specificity of hybridization of the E58 probe is demonstrated in the experimental control shown in Fig. 1, bottom. E58 hybridizes to DNA from a homozygous DRw11 cell line but does not hybridize to DNA derived from a control panel of cells expressing other HLA-DR alleles. The presence of a DRw11 subtype in the patient is also indicated by hybridization with the D70 probe. This probe hybridizes with HLA-DRw8 and with the -DRw11a allele (see control panel in Fig. 1, bottom), thus indicating that the patient is DRw11a. This allele is also present in the patient's father and paternal grandfather. A similar process can be used to interpret the hybridization results using the other two probes. Probe L30



B

DR TYPE	SOURCE	D70	A70	E58	L30
DR 1	WT 100				
DR16, W21	WJR				
DR15, W2	AMAI				
DR16, W22	RML				
DR3, 52a	STEIN				
DR4, W4	WTS				
DR4, W10	YAR				
DR4, W14	PE 117				
DR11, W5, 52b	SWEIG				
DR13, W19, 52c	EMJ				
DR14, W9, 52b	TEM				
DR7, W17	MOU				
DR4, 8	JON				
DR9, W23	DKB				

Figure 1. SSOPH analysis of amplified DNA. (*Top*) An autoradiogram of SSOPH analysis of patient (child) and family members is shown. Each row shows hybridization results derived from the source of the DNA that is listed at the left. The DNA in each column has been hybridized with the oligonucleotide probe indicated at the top of the column. (*Bottom*) The autoradiogram shows hybridization controls using DNA derived from well characterized cells whose name, DR and Dw type are given at left. All cells except JON and DKB are from the core LCL panel from the Tenth International Histocompatibility Workshop. Oligonucleotide probes used in hybridizations are indicated at the top of each column.

hybridization with patient and maternal DNA indicates the presence of DR7. The A70 probe indicates the presence of an HLA-DR2 allele, which is detected in the aunt, half-sibling, maternal grandmother, and paternal grandmother. As shown in Fig. 1, bottom, all hybridization experiments include controls to confirm the specificity of hybridization. A summary of the hybridization data from the HLA-DR, -DQ, and -B loci is presented in Fig. 2. Combining the patient and family data, the class II haplotypes of the patient were deduced as DRw11a(w52b);DQw7/DR7(w53);DQw2. These haplotypes

PROBE	HYBRIDIZATION										ASSOCIATION
DR Locus	C	M	F	mGM	mGF	pGM	pGF	1/2S	A	D1	D2	
E58	+	−	+	−	−	−	+	−	−	+	+	DR5 (w11)
L30	+	+	−	−	+	−	−	−	−	+	+	DR7
D70.1	+	−	+	−	−	−	+	−	−	+	+	DR5 (w11), DR8
H30	+	−	+	−	−	−	+	−	−	+	+	DRw52b
F37	+	+	−	−	+	−	−	−	−	+	+	DR7, DRw52c, DR6Dw9
DE70	−	−	−	+	−	−	−	−	−	−	−	DR6 (w13); DR4Dw10; DRw11b
FL38	−	−	−	+	−	−	+	−	−	−	−	DRw52a
ErCV28	−	−	−	−	−	−	−	+	−	−	−	DR1
A70	−	+	−	+	+	+	−	+	+	−	−	DR2 (w15, w16)
N77	−	−	−	−	−	−	+	−	−	−	−	DR3
H33	−	−	+	−	−	+	−	−	−	−	−	DR4
NII 26	+	+	+	−	+	+	−	−	−	+	+	DRw53
dadvge41	+	+	+	+	+	+	+	+	+	+	+	DR beta
DQ Locus												
E45	+	−	+	n	−	+	+	n	n	+	−	DQw7
EF46	+	+	−	n	+	−	+	n	n	+	+	DQw2
iynre31	+	+	+	n	+	+	+	n	n	+	+	DQ beta
B Locus												
A46	+	+	−	−	+	−	−	−	−	+	+	B13
egpeyw55	+	+	+	+	+	+	+	+	+	+	+	A and B

Figure 2. Summary of SSOPH data from patient, family members, and unrelated donors. The source of the samples is listed across the top (C-patient; M-mother; F-father; mGM-maternal grandmother; mGF-maternal grandfather; pGM-paternal grandmother; pGF-paternal grandfather; 1/2S-half sibling; A-aunt, and D1 and D2-unrelated donors). The probes for each locus are indicated at the left. The association of each probe with known HLA alleles is shown at the right. Boxes indicate the best match between patient and potential donors. With the probes used, potential unrelated donors showed an identical polymorphism profile for HLA-DR, but only donor 1 (D1) was identical for all three loci.

are consistent with established linkage disequilibrium for the caucasian population.

Samples from the patient's family members were typed by both serological and SSOPH methods. The oligonucleotide hybridization data correlated perfectly with the HLA types identified serologically (Figs. 2 and 3). In addition, the serological typing of the patient's family and analysis of the segregation of HLA allowed determination of the haplotypes of the patient's family (Fig. 3). None of the family members were HLA-identical to the patient (Fig. 3). As a result, a search for a well-matched unrelated donor was initiated. The search revealed two potential donors who were HLA-typed by both serological and SSOPH methods. This information was utilized to select the best possible donor for transplantation. The serological typing of the selected donor was A2,11;B13,35,(w4,6);DR7,w11,(w52,w53);DQw2,w7, and the SSOPH pattern was associated with B13;DR7,w11a,w52b,w53;DQw2,w7 (Fig. 2). The patient was transplanted and engrafted readily as determined by chromosomal analysis, normal class I and II HLA expression, and detection of HLA-A11. The patient is presently alive, 39 wk after transplantation and is infection free. She is developing normally with resolving mild/moderate (skin, liver) graft-vs.-host disease.

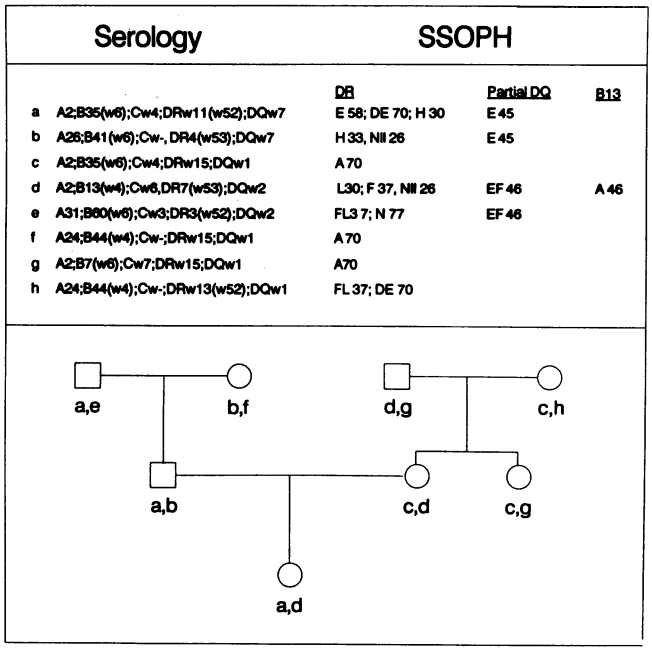


Figure 3. Haplotypes of patient and family members determined by serological and SSOPH analysis. Serological specificities (*top left*) and SSOPH (*top right*) are indicated for each haplotype derived from the pedigree of patient's family (*bottom*).

Discussion

The use of DNA amplification and SSOPH to accomplish HLA matching for transplantation is demonstrated in this study. Although limited use of these techniques has been reported for purposes of disease association and forensic identification, this is the first example of comprehensive HLA typing and subsequent use of the typing for HLA matching in BMT. This new approach for HLA matching provides enhancements that will have great utility in selection of donors for BMT. One important advantage provided by this approach is latitude in quality, quantity, and source of tissue for analysis. This will be valuable in numerous cases in which it is not possible to obtain a sufficient number of normal lymphocytes (e.g., as a result of HLA deficiency or chemotherapy) to achieve reliable HLA typing by conventional methods. A second major advantage of SSOPH methodology is explicit definition of alleles. At this time, serological reagents cannot provide discrimination between all known HLA alleles. In contrast, SSOPH can detect all polymorphism at the level of single amino acid residues. Such micropolymorphism may influence the outcome of transplantation; thus matching at this level may be very beneficial.

The case presented here demonstrates the usefulness of DNA amplification and SSOPH in matching for BMT. In an HLA-deficient SCID patient, serological HLA typing of freshly prepared cells failed to detect most HLA products. Mitogen/lymphokine treatment was unable to induce detectable levels of HLA-B, -DR, or -DQ products. However, analysis of the patient's DNA by SSOPH, which is not dependent upon protein expression, was possible. The combined utilization of serological and SSOPH analysis allowed detection of HLA-A2;B13;Cw4,w6;DR7,w11a,w52b,w53;DQw2,w7. B35 was not tested for, but is predicted by the haplotypes of family members. It was not possible to test for the presence of the B35 allele because the sequence of this allele has not yet been determined.

SSOPH identifies relevant HLA polymorphism and detects single base pair differences that correspond to single amino

acid differences in the proteins. This is a marked improvement over another method of DNA analysis that detects restriction fragment length polymorphism (RFLP). Most of the RFLP results from polymorphism in noncoding sequences of the genome. Therefore, RFLP does not directly measure functional polymorphism, but relies upon linkage disequilibrium with the coding region (4). RFLP analysis has been used for typing a SCID patient (25), but it is less amenable to routine typing because it is labor intensive. Furthermore, RFLP data from heterozygous individuals are difficult to interpret (26).

An important asset of SSOPH methodology is the high level of resolution of HLA alleles obtained. In the case presented here, SSOPH was able to distinguish between three possible types of DRw11 alleles (14, 15) and three possible types of DRw52 alleles (18, Gorski, J., unpublished observations). This level of resolution was useful in donor selection because it confirmed that both donor and recipient shared the same subtypes, DRw11a and DRw52b. Matching with a higher degree of specificity should result in less graft rejection and reduction in frequency and severity of GVHD.

Definition of HLA polymorphism at the level of single amino acid differences provides the basis for a functionally relevant typing system. By retrospective and ongoing SSOPH analysis of BMT donor/recipient pairs, each polymorphism can be evaluated for its effect on the outcome of transplantation. In addition, each polymorphism can be correlated with the extent of serological and cellular alloreactivity. Finally, each polymorphism can be mapped onto the recently elucidated HLA structure (27, 28). This information can be combined to generate a scale rating each position according to its functional importance. Allowable mismatches may be identified using this system, and use of this information in donor selection could ultimately increase the number of successful unrelated donor transplants.

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