Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 1644–1648, March 1987
Immunology

Restriction fragment length polymorphism of the HLA-DP subregion and correlations to HLA-DP phenotypes

(multigene family/major histocompatibility complex/evolution)

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Communicated by Hans Ussing, October 16, 1986 (received for review July 10, 1986)

ABSTRACT **The restriction fragment length polymorphism (RFLP) of the class II HLA-DP subregion of the major histocompatibility complex (MHC) of humans has been unraveled by Southern blotting using DP_α and DP_β probes in a study of 46 unrelated individuals with known HLA-DP types. Contrary to earlier preliminary findings with a limited number of enzymes, the RFLP appears to be quite extensive both with the DP_β (14 different DNA markers defined by individual fragments or clusters thereof) and the DP_α (8 markers) probes, especially when enzymes recognizing only four base pairs were used. A few markers were absolutely or strongly associated with individual DP antigens, whereas most were associated with two or more DP antigens as defined by primed lymphocyte typing. Thus, Southern blotting seems feasible for typing for most DP determinants by specific fragments or subtraction between the various more broadly reactive DNA markers, and the RFLP provides further information on the DP subregion in addition to that provided by primed lymphocyte typing. In two recombinant families, the DP_β and DP_α DNA markers segregated with DP antigens, whereas the DR_β, DQ_β, DQ_α, and DX_α markers followed the DR and DQ antigens.**

The major histocompatibility complex (MHC) of humans, the HLA system, controls three series of class II molecules that are involved in the immune response to various antigens and in susceptibility to various diseases. The series are the HLA-DR and -DQ alloantigens, which can be recognized by serological methods (1), and the HLA-DP antigens, which at present can be detected only by cell culture techniques (primed lymphocyte typing, PLT) (2, 3).

The genetic structure of the class II region of the HLA complex has been partially unraveled in a few haplotypes by the methods of molecular biology (4–7). The restriction fragment length polymorphism (RFLP) of the DR and DQ (including the DX = DQ_2) subregions has been studied extensively (e.g., refs. 7–10), while little is known about the RFLP of the DP subregion. The DP genes control at least seven different PLT-defined alloantigens: HLA-DPw1–6 (2, 3) and CPD-HEI (11).

We have investigated the RFLP of the HLA-DP subregion by means of Southern blotting using DNA probes encoding the DP_α and DP_β genes and compared the presence of various polymorphic DNA fragments with the DP phenotypes established by PLT in a panel of unrelated individuals. Contrary to earlier expectations (7), the RFLP of the DP region appeared to be quite extensive, and some of the fragments were strongly associated with certain DP antigens, suggesting that Southern blotting may be used as an alternative to DP typing with PLT. In addition, we studied the RFLP of the DR, DQ, DX, and DP subregions in two families in which recombinations had occurred within the class II region in an attempt to map more precisely the various class II genes.

MATERIALS AND METHODS

Subjects. A panel of 46 unrelated HLA-DR- and DP-typed individuals was selected for this study. In addition, two families with recombinations between HLA-DR and -DQ on one side and HLA-DP and *GLO* (the gene for glyoxalase) on the other were studied (12, 13).

Detection of Restriction Fragments by Using the Southern Blotting Technique. DNA was extracted from peripheral blood cells as described by Böhme *et al.* (14). Restriction enzyme digests were prepared overnight using 5 units of enzyme per μg of DNA. The enzymes were *Bgl* II, *Eco*RI, *Eco*RV, *Hae* III, *Hin*dIII, *Hin*fI, *Mbo* I, *Msp* I, *Pst* I, *Pvu* II, *Rsa* I, and *Taq* I (= *Tth*HB8I). The digested samples were loaded on agarose gels (0.5–1.5%) and electrophoresed for 25–35 hr at 20–30 V in Tris acetate buffer, pH 8.0. The DNA was then transferred onto Hybond-N nitrocellulose filters (Amersham). Hybridization of the filters was for 40 hr at 42°C in plastic bags with a total volume of 20 ml of 50% (vol/vol) formamide, 5× SSPE (1× SSPE = 180 mM NaCl/10 mM NaH_2PO_4/1 mM EDTA), 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 5% (wt/vol) dextran sulfate, and the radiolabeled probe at approximately 10 ng/ml. After hybridization, the filters were washed twice, 5 min each, in 2× SSPE/0.5% $NaDodSO_4$ (sodium dodecyl sulfate) at room temperature and twice, 30 min each, in 0.2× SSPE/0.5% $NaDodSO_4$ at 65°C. Autoradiography was for 1–7 days on Kodak XAR-5 film.

The DNA probes used were cDNA clones complementary to DR_β, DQ_α, DQ_β, and DP_β chain genes, and a 5-kilobase (kb) genomic *Hin*dIII subclone (subclone 412-1 in ref. 5) complementary to the $DP_{\alpha 1}$ gene. The filters were hybridized primarily with the DP_β cDNA probe pII-β-7, complementary to all exons except the signal sequence and the first half of 1. domain (15) and the genomic probe complementary to the $DP_{\alpha 1}$ gene. β and α probes do not cross-hybridize with α and β genes, respectively.

To ensure that hybridization was not due to cross-hybridization to the genes of the DR or DQ loci, we also hybridized the filters with cDNA probes complementary to the DR_β, DQ_α, and DQ_β chain genes. Only fragments hybridizing exclusively or clearly stronger with the DP_α or DP_β chain probes than to the DR or DQ probes were assigned to the DP subregion.

PLT. PLT was performed by using two different bulk-expanded PLT cells typing for each of the antigens DPw1–6 (2, 3) and CDP-HEI (11).

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Abbreviations: MHC, major histocompatibility complex; PLT, primed lymphocyte typing; RFLP, restriction fragment length polymorphism.

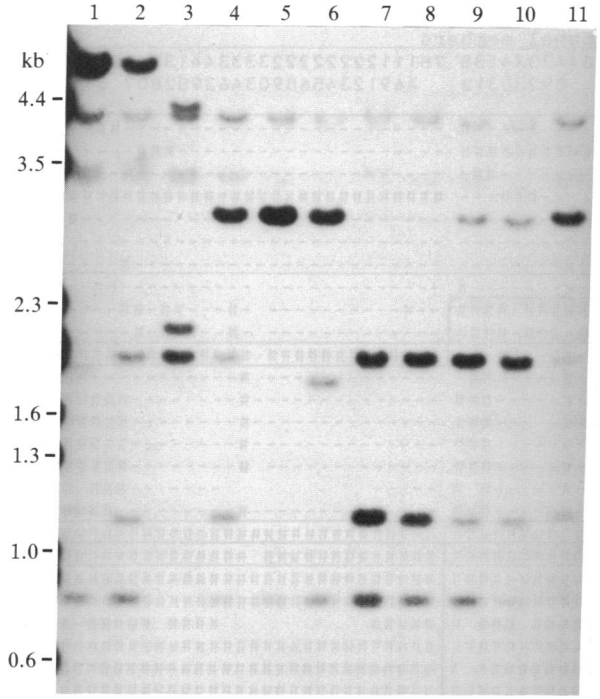

FIG. 1. DP_β-hybridization of *Msp* I-digested DNA. The numbers on top of the lanes correspond to the panel numbers in Fig. 2. Size markers were a mixture of *Hin*dIII- and *Eco*RI/*Hin*dIII-digested bacteriophage λ DNA.

HLA-DR and -DQ Typing. B-lymphocyte-enriched suspensions were typed by the method agreed upon for the 7th International Histocompatibility Workshop (16).

Data Analysis. The polymorphic DNA fragments were first correlated to each other to define clusters of highly correlated fragments. Consensus patterns from these clusters and single fragments not included in clusters defined DNA markers that were then correlated with the HLA-DP antigens. The correlations were estimated as the coefficient of correlation [$r = (\chi^2/N)^{1/2}$] and tested for significance by Fisher's exact test. The P values were corrected for multiple comparisons by multiplication of all P values by the number of comparisons made. A total of 22 polymorphic DNA markers were defined and seven DP antigens were investigated. Thus, all P values obtained in comparing a DNA pattern with a DP antigen should be multiplied by $22 \times 7 = 154$. In some cases, a polymorphic DNA pattern correlated to a group of two or more DP antigens. Such groups can, however, be combined in multiple ways. For example, from the seven DP antigens, two can be combined in $7!/[(7-2)!2!]$ or 21 different ways, which gives a correction factor of $22 \times (7 + 21) = 616$, while it is 1386 for three, 2156 for four, and 2618 for five antigens.

RESULTS

Panel Studies with DP_β Probe. Nine of the 12 enzymes used gave rise to polymorphic fragments (Fig. 1) when hybridization was carried out with the DP_β probe. Fig. 2 shows the polymorphic DP_β fragments in 46 unrelated individuals. Twenty-one of the fragments could be grouped into five clusters consisting of between two and six fragments. Nine fragments could not be clustered.

The clusters and individual fragments defined 14 DNA markers arbitrarily numbered from 1 to 14. Eleven of these markers correlated with individual DP antigens or combinations thereof (Fig. 2, Table 1). Single and double intensity of bands indicating hetero- and homozygosity was taken into account when assigning various DP antigens to a cluster (see the double intensity of the 1.9-kb *Msp* I fragments in lanes 3, 7, 8, 9, and 10 in Fig. 1). The *Msp* I 4.9-kb fragment (marker 1) was absolutely associated with the DPw1 antigen, marker 2 was strongly associated with DPw2, and the *Msp* I 1.1-kb fragment (marker 3) included the DPw4 antigen (Fig. 2). Clusters 4 to 11 included various combinations of DP antigens. All except the associations of cluster no. 11 were significant after correction of P values. Note the partial complementarity of markers 5 and 9 and of 10 and 11.

Panel Studies with DP_α Probe. Hybridization with the genomic probe encoding the $DP_{\alpha 1}$ gene resulted in between one and four polymorphic fragments per enzyme—i.e., 20 polymorphic fragments, 15 of which could be ordered in three clusters (Fig. 2). The *Msp* I 6.5-kb fragment (marker 15) correlated strongly with DPw1 + -w5 (Table 1). Markers 16 and 17 correlated with DPw5 and DPw2, respectively.

Table 1. Correlations between RFLP with DP probes and HLA-DP antigens

Probe	Marker	Representative fragment	HLA-DPw*	Fragment versus DP ++	+−	−+	−−	N	P	Correction factor	Corrected P	r
DP_β	1	*Msp* I 4.9	1	4	0	0	41	45	7×10^{-6}	154	1×10^{-3}	1.00
	2†	*Rsa* I 1.7	2	9	5	1	30	45	2×10^{-5}	154	3×10^{-3}	0.68
	3	*Msp* I 1.1	4	31	3	0	11	45	4×10^{-8}	154	6×10^{-6}	0.85
	4	*Msp* I 3.0	3 + 5 + 6	14	2	0	29	45	7×10^{-10}	1386	1×10^{-6}	0.90
	5	*Pst* I 2.9	1 + 3 + 5 + 6 + H	19	2	0	25	46	1×10^{-10}	2618	3×10^{-7}	0.92
	6	*Bgl* II 20.0	1 + 3 + 5 + 6	18	2	0	26	46	1×10^{-10}	2156	2×10^{-7}	0.91
	7	*Rsa* I 2.1	1 + 2 + 4 + 5	42	0	0	4	46	6×10^{-6}	2156	1×10^{-2}	1.00
	8	*Msp* I 1.9	2 + 4 + 5	40	1	0	4	45	3×10^{-5}	1386	4×10^{-2}	0.88
	9	*Pst* I 2.4	2 + 4	39	1	0	6	46	8×10^{-7}	616	5×10^{-4}	0.91
	10	*Hin*dIII 5.0	2 + 3 + 6 + H	18	1	8	19	46	8×10^{-6}	2156	2×10^{-3}	0.65
	11	*Hin*dIII 5.3	1 + 4 + 5	34	3	2	7	46	7×10^{-5}	1386	NS	0.67
DP_α	15	*Msp* I 6.5	1 + 5	7	0	1	35	43	2×10^{-7}	616	1×10^{-4}	0.92
	16	*Taq* I 6.5	5	3	0	1	37	41	4×10^{-4}	154	6×10^{-2}	0.85
	17	*Taq* I 13.5	2	8	1	2	32	43	3×10^{-6}	154	5×10^{-4}	0.80
	18	*Bgl* II 3.5	1 + 5 + H	9	6	0	31	46	5×10^{-6}	1386	7×10^{-3}	0.71
	19	*Bgl* II 2.3	2 + 3 + 4 + 6	41	0	0	3	44	8×10^{-5}	2156	NS	1.00
	20	*Pst* I 4.3	6	2	2	1	40	45	2×10^{-2}	154	NS	0.54

Individuals were typed for HLA-DPw1–6 and CDP-HEI with the PLT technique. Fragments are identified by the endonuclease and size in kb. N is the number of individuals included in the calculation. The correction factor is the number of comparisons made (see text); r is the coefficient of correlation. NS, not significant.

*H is the CDP-HEI specificity (11).

†The fragment with broadest reactivity in a cluster was chosen for the calculations.

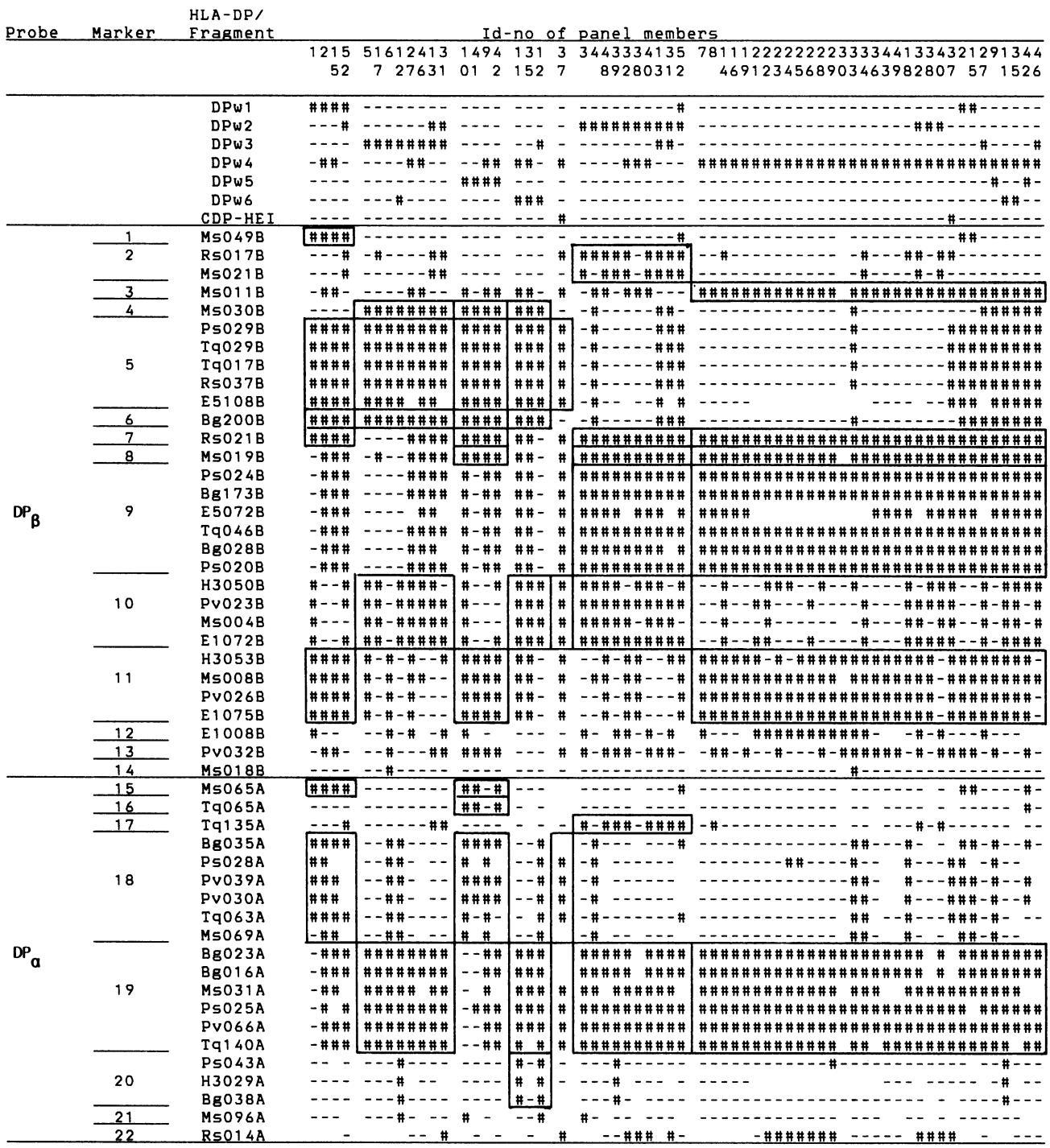

FIG. 2. HLA-DP fragmentogram. Distribution of seven DP antigens, 30 DP_β, and 19 DP_α allogenotypes in 46 unrelated individuals. Id-no, identification number. (Note that these numbers are printed vertically—e.g., $\frac{1}{3}$ is 15.) Rows indicate allogenotypes defined by a code word in which the two first positions indicate the enzyme and the next the size of the fragment in kb multiplied by 10; e.g., Ms049 indicates DNA digested with *Msp* I, producing a 4.9-kb fragment. Bg, *Bgl* II; E1, *Eco*RI; E5, *Eco*RV; H3, *Hin*dIII; Ms, *Msp* I; Ps, *Pst* I; Pv, *Pvu* II; Rs, *Rsa* I; Tq, *Taq* I. A and B refer to the DP_α and DP_β probes, respectively. Columns correspond to panel members ordered after DP assignments. Individuals with two DP antigens are included twice. The presence of a fragment is indicated with #, absence with -; no symbol indicates not done.

Marker 18 included DPw1, -w5, and CDP-HEI and seemed complementary to the broadly reactive marker 19, which was absolutely but insignificantly associated with DPw2, -w3, -w4, and -w6. Marker 20 might be associated with DPw6.

It should be noted that most of the discrepancies (+− and −+ in Table 1) were subjected to repeated RFLP studies and PLT investigations, and in all cases they appeared to be reproducible. In addition to the polymorphic bands, the DP_α probe gave rise to 6 and the DP_β probe to 31 nonpolymorphic bands with the enzymes used.

Family Studies. Two families with DR/DP recombinants were studied with the restriction enzymes *Hin*dIII, *Pst* I, and *Taq* I (the GG family also with *Bgl* II, *Eco*RV, and *Pvu* II), and the class II probes DR_β, DQ_α, DQ_β, DP_α, and DP_β.

The upper part of Table 2 shows the segregation in the BN family of the fragments detected by the DR_β, DQ_α, DQ_β, and DP_β probes. The fragments obtained with the DP_α probe were not informative in this family. The individual fragments could be grouped into a number of patterns with different segregations. For example, pattern 1 for the DR_β probe included

Table 2. Segregation patterns in the BN and GG families

Family	Individual	Haplotypes	DR	DQw	DPw*	*GLO*	RFLP patterns: DR_β 1	2	3	4	5	6	DQ_β 1	2	3	4	5	DQ_α 1	2	3	4	5	DP_β 1	2	3	DP_α 1	2
BN	Father	*a*	4	3	6	1	+	+	−	−	+	+	+	−	−	+	+	+	−	+	+	+	+	+			
		b	2	1	4	2																					
	Mother	*c*	7	2	4	1	−	−	+	+	+	+	−	+	+	+	+	−	+	+	+	++	−	++			
		d	w6	—	—	1																					
	1st child	*a* × *b*†	2	1	6	1	−	+	+	−	+	++	+	+	−	+	+	+	−	+	+	+	+	+			
		c	7	2	4	1																					
	2nd child	*a*	4	3	6	1	+	−	+	−	++	+	−	+	−	++	−	−	−	++	−	++	+	+			
		c	7	2	4	1																					
	3rd child	*b*	2	1	4	2	−	+	+	−	+	++	+	+	−	+	+	+	−	+	+	+	−	++			
		c	7	2	4	1																					
	4th child	*b*	2	1	4	2	−	+	+	−	+	++	+	+	−	+	+	+	−	+	+	+	−	++			
		c	7	2	4	1																					
											a	*b*				*a*	*b*			*a*	*b*	*a*		*b*			
		Segregation with haplotypes:					*a*	*b*	*c*	*d*	*c*	*c*	*b*	*c*	*d*	*c*	*d*	*b*	*d*	*c*	*d*	*c*	*a*	*c*			
																						d		*d*			
GG	Father	*a*	2	1	4	2	+	+	−	++	+		+	−	++			+	+	−	++		+	+	+	+	+
		b	6	1	H	1																					
	Mother	*c*	2	1	4	2	+	−	+	+	++		−	+	+			+	−	+	+		+	−	++	−	++
		d	—	1	2	2																					
	1st child	*b*	6	1	H	1	+	+	−	++	+		+	−	++			+	+	−	++		+	+	+	+	+
		c	2	1	4	2																					
	2nd child	*b* × *a*†	6	1	4	2	−	+	+	++	+		+	+	+			−	+	+	+		+	−	++	−	++
		d	—	1	2	2																					
	3rd child	*a*	2	1	4	2	+	−	+	+	++		−	+	+			+	−	+	+		+	−	++	−	++
		d	—	1	2	2																					
	4th child	*b*	6	1	H	1	+	+	−	++	+		+	−	++			+	+	−	++		+	−	+	+	+
		c	2	1	4	2																					
	5th child	*b* × *a*‡	6	1	H	2	−	+	+	++	+		+	+	+			−	+	+	+		−	+	+	+	+
		d	—	1	2	2																					
							a			*b*	*a*				*a*§			*a*			*a*§		*a*		*a*		*a*
		Segregation with haplotypes:					*c*	*b*	*d*	NP	*c*		*b*	*d*	*b*			*c*	*b*	*d*	*b*		*c*	*b*	*c*	*b*	*c*
											d				*c*						*c*				*d*		*d*

The patterns obtained in the two families with the five class II probes consist of between 1 and 14 polymorphic fragments. The two DX_α specific *Taq* I fragments of 2.2 and 2.1 kb obtained with the DQ_α probe in the BN family are included in patterns 5 and 1, and in the GG family in patterns 4 and 3, respectively. The parents of the BN family are included in Fig. 2, having identification nos. 35 (father) and 36 (mother), and the parents of the GG family have identification nos. 37 (father) and 38 (mother). Bands with double intensity suggesting double gene dose are indicated with ++. NP, not polymorphic.

*H is the CDP-HEI specificity.

†Recombination in the HLA-D region.

‡Recombination between HLA-DP and *GLO*.

§These patterns consist of a combination of the *a*, *c*, and *b* haplotypes, but it is impossible to decide whether these patterns are DR- or DP-like as both combinations are possible.

seven different fragments segregating with the paternal *a* haplotype, while pattern 4 included eight fragments segregating with the maternal *d* haplotype. In some cases, homozygosity for a given fragment was revealed by double intensity of the corresponding band on the Southern blot. This is, for example, true of the fragments of pattern 2 of DP_β, which segregated with DPw4 in the *b*, *c*, and *d* haplotypes and thus were present in double doses in the mother, child 3, and child 4. For each of the four probes, the father was heterozygous for at least one pattern not present in the mother, and the father was, thus, fully informative in terms of recombination. The informative DR_β, DQ_α, and DQ_β patterns all followed the segregation of the paternal HLA-DR and -DQ antigens, whereas pattern 1 for the DP_β probe followed the DPw6 antigen. Thus, the recombination must have taken place between the DR_β, DQ_α, and DQ_β genes on the one hand and the DP_β genes on the other. As discussed below, the 2.2- and 2.1-kb *Taq* I fragments obtained with the DQ_α probe represent the DX_α gene which, thus, in this family can be mapped to the DR and DQ side of the recombination.

The lower part of Table 2 shows the segregation patterns detected by the five class II probes in the GG family. The segregations of the hybridizing fragments were informative for the DR, DQ, and DP subregions, but not for the DX locus. In child 2, a recombination had occurred between HLA-DR + DQ and HLA-DP. The RFLP patterns of DQ_α, DQ_β, and DR_β followed HLA-DRw6 (the *b* haplotype), while the DP_α and DP_β RFLP followed HLA-DPw4 (the *a* haplotype) in the recombinant HLA haplotype of child 2. Thus, the recombination has taken place between the DR_β, DQ_β, and DQ_α loci on the one hand and the DP_β and DP_α loci on the other. Child 5 carries a recombination between HLA-DP and *GLO*. All RFLP markers followed HLA-DR, -DQ, and -DP of the recombinant haplotype and, thus, the recombination has taken place centromeric to all HLA genes investigated.

DISCUSSION

This study shows that the RFLP of the HLA-DP subregion is considerably more pronounced than previously assumed (7). We identified 14 different DNA markers by clusters of fragments or individual fragments when hybridizing with a DP_β probe and 8 different markers when hybridizing with a

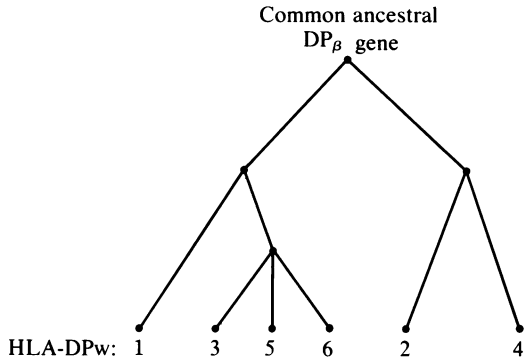

FIG. 3. Proposed evolution of the DP_β gene polymorphism.

DP_α probe (Fig. 2). The pronounced DP polymorphism was only seen because we also used restriction enzymes recognizing four base pairs. Moreover, our panel of cells was selected on the basis of DP types. The RFLP of DP is more extensive than presently recognizable for the gene products at the cell surface, where only eight different DP antigens and a few splits can be detected (11). Some of the polymorphism of the DNA markers may, however, be due to noncoding sequences, which will not be reflected in the gene products.

The observations in Fig. 2 and Table 1 indicate that RFLP studies represent an alternative to the PLT test in DP typing because most of the RFLP markers were significantly and usually very strongly associated with one or more DP antigens. It appears that, apart from DPw3 and -w6, all the other DP determinants can be distinguished on the basis of their RFLP patterns and that all this information can indeed be detected by just three enzymes—*Msp* I, *Taq* I, and *Bgl* II—and the DP_β probe. However, only a few of the associations were absolute ($r = 1.00$). Most of the discrepancies were due to the presence of the RFLP marker in the absence of the corresponding DP antigens(s). It seems unlikely that the discrepancies were caused by technical errors of DNA or DP typing because they were confirmed by repeated investigations. Indeed, we have preliminary evidence that some of the "extra reactions" seen at the DNA level are due to additional associations with low-frequency DP antigens not included in the present study. Thus it seems likely that all the associations between RFLP markers and DP antigens will eventually become absolute when more DP antigens are defined. At the present time, more information is obtained by RFLP than by PLT studies because there are more DNA markers than DP antigens known. Moreover, RFLP results may serve as a guide in selecting responder/stimulator combinations in the search for new PLT reagents.

Kappes *et al.* (17) showed that two polymorphic *Hin*dIII fragments of 5.3 and 5.0 kb were related to the $DP_{\beta 2}$ pseudogene. We found three fragments strongly associated with the 5.0-kb *Hin*dIII fragment and three other fragments strongly associated with the 5.3-kb *Hin*dIII fragment. These two clusters of fragments (markers 10 and 11) most probably represent alleles of the $DP_{\beta 2}$ pseudogene. With several enzymes, the DP_α probe revealed only one band, suggesting that the DP_α probe hybridizes only to the $DP_{\alpha 1}$ gene and not to the $DP_{\alpha 2}$ pseudogene. Hybridization at low stringency (washing with 2× SSPE/0.5% $NaDodSO_4$ at room temperature) did not result in any detectable hybridization to the $DP_{\alpha 2}$ gene (data not shown).

The DP associations of markers 1–9 and the complementarity of the markers 5 and 9 (Fig. 2) may indicate that a common ancestral $DP_{\beta 1}$ gene was first split in two branches, from one of which the DPw2 and -w4 genes subsequently developed, whereas the other developed into DPw1 and another branch from which DPw3, -w5, and -w6 originated (Fig. 3). The complementary pattern of the $DP_{\beta 2}$ pseudogene markers 10 and 11 is strikingly similar to that of the $DP_{\alpha 1}$ markers 18 and 19, which is extraordinary because these genes are located at the extreme end of the DP subregion (5, 7).

The observations in the two families with intra-class II region recombinants comfirmed that the DP_α and DP_β genes map centromeric to both the DR and DQ subregions. Spielman *et al.* (18) and Le Gall *et al.* (19) reported that the RFLP pattern of the DX genes formed two allelic clusters represented by, e.g., the two *Taq* I fragments of 2.2 and 2.1 kb, respectively. The GG family showed that these alleles followed the DR and DQ genes, demonstrating that the DX locus was on the DR side of the recombination (Table 2).

We thank Mrs. Ingrid Alsing, Mrs. Ewa Szojmer, Mrs. Isabella Wang Hansen, and Miss Jannie Hansen for skillful technical assistance, and Mrs. Birgitte Kjærsgaard for secretarial assistance. The probes used in this study were kindly provided by L. Rask and P. A. Peterson, Uppsala. J.J.H.-N. is the recipient of a grant from Løvens kemiske Fabrik (Leo, Denmark), and the work was supported by The Danish Multiple Sclerosis Society, the Danish Medical Research Council, and The Danish Rheumatism Association.

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