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Genetics

Six HLA-D region α-chain genes on human chromosome 6: Polymorphisms and associations of DCα-related sequences with DR types

(linkage disequilibrium/restriction fragment length polymorphism)

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ABSTRACT **Analysis of cosmid clones containing genes related to the HLA-DRα chain calls for at least six HLA-D region α-chain coding sequences in man; namely, DRα, DCα, DXα (very closely related to DCα), SBα1, SBα2 (two closely linked genes on the same cosmid clones), and DZα. The first four genes have been described previously. SBα2 and DZα are recently identified genes, characterized by their unique and, from a limited study, nonpolymorphic bands when used as probes for human DNA on Southern blots. All of the genes are present in somatic cell hybrids containing a human X/6 translocation chromosome, and so they are all presumably in the HLA region. The polymorphisms in the region of the DCα and related DXα genes were studied with Southern blots of DNA from a set of mostly homozygous HLA-D-typing cell lines. With *Eco*RI, the band patterns for the DCα gene corresponded to the major cross-reactive HLA-DR serotypes associated with DC (namely MT1, -2, and -3) while the DXα band was invariant. Both genes were polymorphic with the enzyme *Taq* I. Within some DR types additional polymorphic variation was detected at the DNA level, implying the existence of subtypes. The pattern of polymorphisms for DCα, and to a lesser extent for DXα, suggests that these genes may play an important role in certain HLA-D associations with disease.**

The HLA-D region (class II) genes determine cell-surface glycoproteins, which function in the immune response. The known products of these genes are heterodimers of an α and a β chain. There are at least three major sets of products coded for by the HLA-D region. These are HLA-DR (homologous to I-E of the mouse H-2 region), defined by conventional serological methods; DC or MT (homologous to mouse I-A), originally defined by the cross-reacting group that included DR1, -2, and -w6; and SB, the "secondary B" cell types defined by primed lymphocyte typing (for reviews, see refs. 1 and 2). A fourth product, closely related to DR and called BR, has also been proposed on the basis of immunochemical analysis (3). Unlike the HLA-A and -B loci, whose original antigens were rapidly "split" to yield more than 20 HLA-A and more than 40 HLA-B alleles, the list of HLA-DR alleles has not yet been greatly expanded.

Studies by two-dimensional gel electrophoresis have shown that most of the serologically detected polymorphic variation is attributable to variation in the β chains of the DR and DC molecules (1, 4), and DNA probes for the β chains have revealed a high level of restriction fragment polymorphism (5, 6). In contrast, probes for the DRα- and SBα-chain genes detect little restriction-site polymorphism among unrelated individuals, while a probe for the DCα chain detects a high level of polymorphism (7, 8). These results are consistent with the finding of sequence variation in regions of cDNA clones for DCα and the homologous I-A α chains in mouse (9, 10). In preliminary studies, the variation detected by the DCα probe appeared to be associated with certain patterns of allelic variation in HLA-DR (7, 8). The other three class II α-chain genes described previously showed little restriction enzyme polymorphism (7, 11).

In this paper we describe two additional class II α-chain gene sequences, both on chromosome 6. We have also studied systematically the variation in restriction patterns in two genes as revealed by the DCα-chain probe. Using a panel of cell lines, that are for the most part homozygous for HLA-DR, there is a strong association, due to linkage disequilibrium, between the restriction patterns and the HLA-DR types. Within some DR types, even those that are serologically homogeneous, there is additional polymorphic variation detected at the DNA level for both the DCα and the related DXα genes.

MATERIALS AND METHODS

Enzymes and Other Reagents. Restriction enzymes were purchased from Cambridge Biotechnology Laboratories and New England Biolabs., radiochemicals were from the Radiochemical Centre, and DNA polymerase I was from Boehringer Mannheim.

Cell Lines. All DNA samples were extracted from the lymphoblastoid lines shown in Table 2. Most of the cell lines behaved as homozygous typing cells in the mixed lymphocyte reaction, which detects mainly HLA-DR-associated determinants. The human–mouse hybrids used for assigning sequences to chromosome 6 contained a human X/6 translocation chromosome from the cell line G3.32.2 (7, 12).

Blotting, Probes, and Hybridization. High molecular weight DNA (7.5–15 μg) from each cell line was digested to completion with restriction endonuclease, then subjected to electrophoresis in 0.7% agarose for 16–20 hr. DNA was transferred to nitrocellulose filters by Southern blotting (13). The DCα-chain sequence used as a probe is the *Pst* I fragment from cosmid LC14 (*Taq* I digests) or LC10 (*Eco*RI digests) (see figure 2 in ref. 7). It was labeled by nick-translation to $\approx 10^8$ cpm/μg (14). Hybridization was carried out as described (7). Filters were washed twice in 2× NaCl/Cit/0.1% $NaDodSO_4$ for 15 min, and twice in 0.2× NaCl/Cit/0.1% $NaDodSO_4$ for 1 hr, both at 65°C (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate). Kodak XAR-5 film was exposed to the filters for 3–7 days at −70°C.

RESULTS

Two New Class II α-Chain Coding Sequences. A series of cosmid clones containing sequences hybridizing to an HLA-

Abbreviations: kb, kilobase(s); NaCl/Cit, standard saline citrate.

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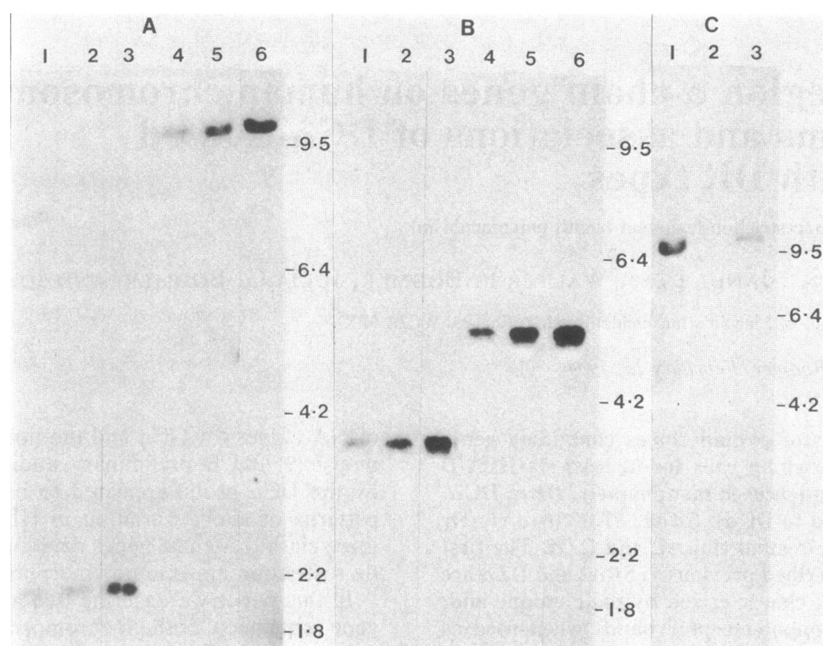

FIG. 1. Demonstration that the DZα and SBα2 genes are contained on unique, characteristic restriction enzyme fragments in human genomic DNA (*A* and *B*, respectively), and mapping of DZα to chromosome 6 (*C*). *Pst* I (lanes 1–3 in *A* and *B*) and *Eco*RI (lanes 4–6 in *A* and *B*; lanes 1–3 in *C*) digested DNA samples (10 μg per lane) were loaded onto 0.7% agarose gels. After electrophoresis at 20 V for 36 hr, the DNA was denatured and then transferred to nitrocellulose sheets. High molecular weight DNAs from the following cell lines were used. *A* and *B*: lanes 1 and 4, G3.32.2 (HLA-DR 2,5); lanes 2 and 5, Maja (HLA-DR 1,1); lanes 3 and 6, Mann (HLA-DR 7,7). *C*: lane 1, GBS1, hybrid with X/6 translocation chromosome; lane 2, GBS1R, segregant of GBS1 differing from it in the absence of the X/6 chromosome; lane 3, G3,32,2 (human parent of GBS1). A hybrid MCP-6, which also contained the X/6 translocation and no other human chromosomes, gave similar results (12). Probes: DZα *Pst* I fragment (*A* and *C*); SBα2 *Pst* I fragment (*B*). Hybridization was with 6× NaCl/Cit/0.1% $NaDodSO_4$ at 65°C, and washing was with 0.2× NaCl/Cit/0.1% $NaDodSO_4$ at 65°C.

DRα chain cDNA probe were described in an earlier publication from this laboratory (7). These clones provided strong evidence for at least four HLA-D α-chain coding sequences on chromosome 6: DRα, DCα and the very similar DXα, and a locus we referred to as DRα-related and suggested might be SBα (7, 11). It has now been confirmed that the sequence of this DRα-related gene closely matches the sequence of an SBα chain cDNA clone, identified as such by homology with the presumed SBα polypeptide sequence, and so we will refer to it provisionally as SBα1 (15, 23). One of the two further class II α-chain coding sequences, SBα2, was identified as an additional distinct sequence hybridizing to the HLA-DRα cDNA probe on cosmids LC11 and JG8a, which contain the SBα1 gene. The justification for calling this sequence SBα2 is based on its tight linkage to SBα1, although we should point out that it can be distinguished from that gene by stringent hybridization (see below). It is also flanked by two SBβ genes (unpublished data). The other gene, DZα, was found on another cosmid clone, JG8b, as a sequence that hybridized to the HLA-DRα cDNA probe, and has not been linked to any other cosmids.

To validate the finding that these α-chain genes did not correspond to DRα, DCα, DXα, or SBα1, Southern blots of human DNA were screened with probes that had been excised from these genes using *Pst* I and subcloned into plasmid pAT153. The subcloned fragment from cosmid LC11 (SBα2) was 3.2 kilobases (kb) long, and that from cosmid JG8b (DZα) was 2.0 kb long. Both of these subclones hybridized to a ^{32}P-labeled DRα cDNA insert probe (16). DNAs from three different human cell lines digested with two different enzymes, *Pst* I and *Eco*RI, were analyzed (Fig. 1). The SBα2 probe gave rise to *Eco*RI and *Pst* I bands of 5.5 kb and 3.2 kb, respectively; the DZα probe corresponded to bands of 10.0 kb and 2.0 kb, all when hybridized under stringent conditions. In each case, these bands matched the sizes of the bands obtained when cosmids containing these genes were probed with the DRα chain cDNA insert, as described above, confirming that the subcloned fragments contained the sequences that were DRα-related. Neither probe revealed any polymorphism with *Eco*RI or *Pst* I on this limited set of cell lines, which, however, included four of the more frequent HLA-DR types (Fig 1). A summary of the six different HLA-D region α-chain genes described so far, and their characteristic band sizes, is given in Table 1.

Mapping to Chromosome 6. It has already been shown that all of the genes listed in Table 1, other than SBα2 and DZα, map to chromosome 6 and thus, most probably, to the HLA region (7). This must also be the case for the SBα2 gene, because it is very closely linked to SBα1 on the same cosmid

Table 1. Summary of characterization of HLA-DRα-related genes on chromosome 6

Gene	Characteristic band sizes in kb: *Eco*RI	*Pst* I	Comments
DRα	3.2, 4.5	2.0, 5.8	
DZα	10.0	2.0	See text and Fig. 1
DCα	Variable (15.5)	Variable (12.5)	See Figs. 2 and 3
DXα	5.0	Variable (6.6)	See Figs. 2 and 3
SBα1	11.0	1.9, 2.3	See Fig. 1; Closely linked (see text)
SBα2	5.5	3.2	Closely linked (see text)

Except in the case of the DCα and DXα genes, the band sizes obtained on Southern blots of human genomic DNA were identical to those present in the cosmid clones (for details, see text). Because of the polymorphism of these two genes, characteristic sizes for a DRw6 cell line are shown. Patterns typical of some other specificities have been described (see Figs. 2 and 3; ref. 7). All the band sizes are approximate, and those >10 kb may not be very accurate.

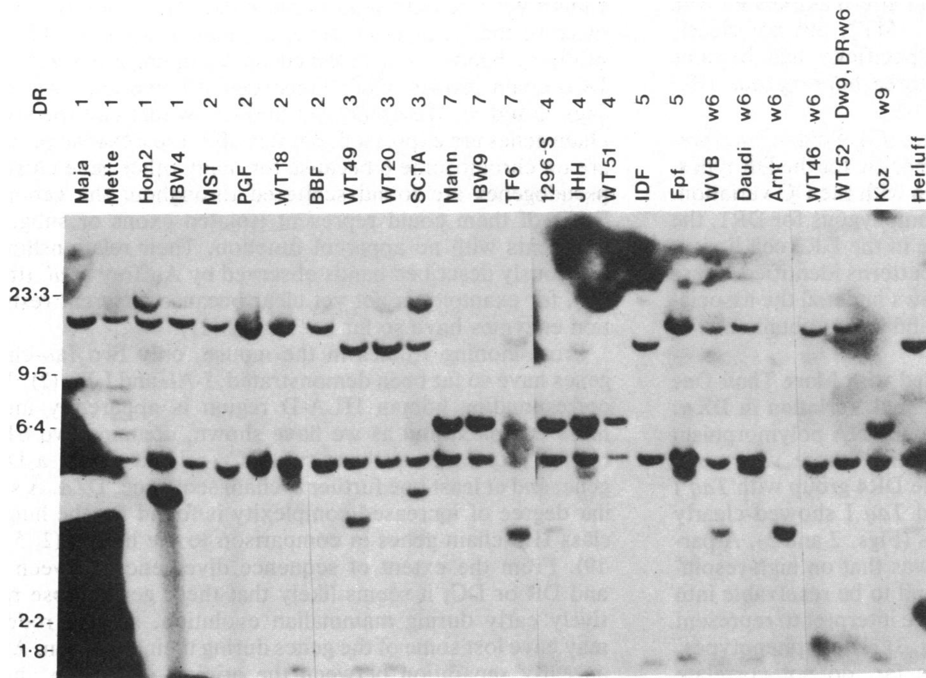

FIG. 2. Southern blot patterns of DNA from DR-typing cell lines digested with *Eco*RI and probed with the DCα-chain DNA probe. The DR types of the cell lines, as determined, or confirmed in our laboratory are given above each line. Size markers were bacteriophage λ digested with *Hin*dIII. The blot was prepared as described in *Materials and Methods* and in the legend to Fig. 1. The data from this blot were used to compile Table 2 (in compiling Table 2, bands that were not consistently present were ignored).

clone. That the DZα gene similarly maps to chromosome 6 is shown in Fig. 1*C*, using somatic cell hybrids containing the part of chromosome 6 that includes the HLA region. Only the X/6-containing human–mouse hybrids react with the DZα clone under stringent conditions, which are such that there is no cross-hybridization with any of the other α-chain sequences.

Highly Polymorphic DNA Restriction Fragments are Detected by the DCα-Chain Probe and are Strongly Associated with HLA-DR Types. The experiments described above indicated that, of the six class II α-chain sequences, only the DCα gene was highly polymorphic, although the DXα fragment had previously shown some variation with *Pst* I (7). DNA was prepared from the cell lines listed in Table 2, representing homozygotes for most of the well-characterized HLA-DR types. Further studies were done using these DNAs with the DCα-chain probe using *Eco*RI (Fig. 2) and *Taq* I (Fig. 3). The results are consistent with the conclusion that there are at least two DCα-like sequences in the haploid genome, which we shall continue to refer to as DCα and DXα, although this does not rule out the possibility of further related sequences.

The patterns detected using *Eco*RI, as indicated previously, correspond with the HLA-DR cross-reactive serotypes (DR1, -2, -w6, DR3, -5; DR4, -7) that are associated with variation at the DC locus. Several new examples of this correspondence were observed. (*i*) KOZ (HLA-DRw9, defined as the DR "4 × 7" cross-reacting group) has the restriction pattern common to DR4 and DR7, (*ii*) WT52 (Dw9 but serological type DRw6) has the DRw6 restriction pattern. Furthermore, the HLA-DR5 cell line FpF, shown unexpectedly to share the DC1 and MT1 (usually DR1, -2, -w6 associated)

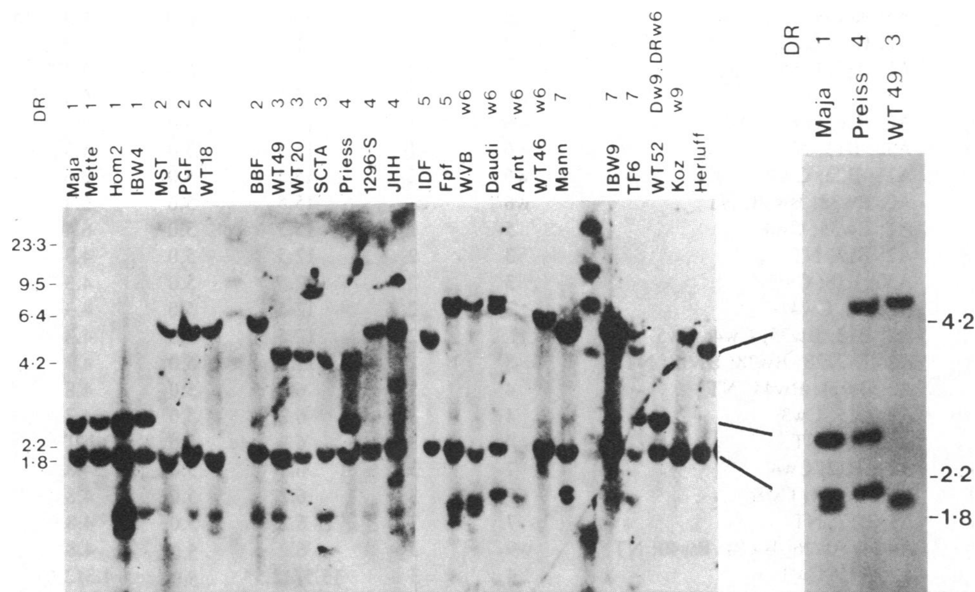

FIG. 3. Southern blot patterns of DNA from the panel of DR-typing cell lines digested with *Taq* I and probed with the DCα-chain DNA probe. Some additional blots are also shown in which a higher resolution was obtained, enabling the doublet of bands at 1.9 kb and 2.0 kb to be distinguished.

serological type had the *Eco*RI band pattern associated with MT1. The cell line Herluf, which is MT2, but not clearly classified with respect to DR or Dw specificity, had the same restriction enzyme pattern as the three homozygous DR3 cell lines, which are also MT2 (Fig. 2).

The position of the larger band after *Taq* I digestion, (presumed to be DCα) was, in general, specific for the DR types, and there was no obvious association with the DC variation. For example, in all four cell lines homozygous for DR1, the variable band size was 2.6 kb, while in the DR2 cell lines it was 5.3 kb, and so on. These *Taq* I patterns identified Priess as heterozygous for DC. Table 2 shows in detail the association of the various HLA-DR types and bands obtained with both enzymes.

Some HLA-DR Types are Associated with More Than One Restriction Fragment Pattern: Additional Variation in DXα. In addition to distinctive differences in DNA polymorphism between HLA-DR and DC specificities, there was some variation within DR specificities. The DR4 group with *Taq* I and the DR5 group with *Eco*RI and *Taq* I showed clearly different restriction enzyme patterns (Figs. 2 and 3). A particular feature of the *Taq* I digests was that on high-resolution gels, the band at 1.9 kb was found to be resolvable into two electrophoretic variants, which we interpret to represent different alleles of the DXα gene (Fig. 3). These phenotypes, from the limited sample studied so far, do not correlate strongly with DR specificities (the three DR7s, for example, show at least two different patterns; Table 2).

DISCUSSION

The two HLA-D region α genes, SBα2 and DZα, described in this paper, bring to six the total number of class II α genes or related subgenic fragments so far identified by us. We cannot yet rule out the possibility that there may be one or more further related sequences, because some weakly hybridizing bands were obtained on Southern blots with the DCα chain probes under nonstringent conditions (see also Figs. 2 and 3). We do not yet know how many of the six α-chain genes are expressed, but it is of interest that all of them are on chromosome 6 because for many other gene clusters pseudogenes are found scattered throughout the genome. Some of them could represent isolated exons or subgenic fragments with no apparent function. Their relationship to previously described bands observed by Auffray *et al.* (their DY, for example) is not yet clear because different restriction enzymes have so far been used (11).

From cloning studies in the mouse, only two Iaα-chain genes have so far been demonstrated, I-Aα and I-Eα (2). The corresponding human HLA-D region is apparently much more complex, and as we have shown, contains two SBα-related sequences, at least two DCα-related genes, a DRα gene, and at least one further α-chain sequence, DZα. A similar degree of increased complexity is found for the human class II β-chain genes in comparison to the mouse (2, 5, 6, 19). From the extent of sequence divergence between SB and DR or DC, it seems likely that these genes arose relatively early during mammalian evolution, so that rodents may have lost some of the genes during their evolution. After an early separation between the original class II α and β chains, the subsequent evolution of SB, DC, and DR probably involved duplication of $\alpha\beta$ combinations followed by further duplication of individual α and β genes within each of these subclusters. Thus, given their sequence similarity, the separation of DCα and DXα and of SBα1 and SBα2 is likely to be comparatively recent and certainly after the divergence of rodents and primates (19, 23). More detailed mapping

Table 2. The correspondence between HLA-DR types and restriction enzyme fragment haplotypes detected by the DCα-chain probe

				*Eco*RI		*Taq* I	
Cell line	HLA-ABC	DR	MT	DC	DX	DC	DX
Maja	A2; Bw35; Cw4	1	1	15.5	5.0	2.6	1.9/2.1
Mette	A2; A3; B5; B18; NT	1	1	15.5	5.0	2.6	a
Hom2	A3; B27; C1	1	1	15.5	5.0	2.6	a
IBW4 (C)	A3; B35; Cw4	1	1	15.5	5.0	2.6	a
MST	A3; B7; NT	2	1	15.5	5.0	5.3	2.1
PGF (C)	A3; B7; NT	2	1	15.5	5.0	5.3	a
WT18 (C)	A2; B27; Cw2	2	1	15.5	5.0	5.3	a
BBF (C)	A1; B37; Cw6	2	1	15.5	5.0	5.3	a
Daudi		w6	1	15.5	5.0	6.4	a
WVB (C)	A2; B16; NT	w6	1	15.5	5.0	6.4	a
WT46 (C)	A32; B13; NT	w6	1	15.5	5.0	5.3	a
WT52 (C)	A11; B22; Cw3	w6	1	15.5	5.0	2.6	a
Arnt	A2; Bw38; Bw39; NT	w6	1	15.5	5.0	6.4	a
FpF (C)	A1; Bw35; Cw4	5	1	15.5	5.0	6.4	a
WT49 (C)	A2; B17; NT	3	2	12.5	5.0	4.3	1.9
WT20 (C)	A30; B18; Cw5	3	2	12.5	5.0	4.3	a
SCTA	A1; B8; Cw4	3	2	12.5	5.0	4.3	a
Herluf	A2; B12; Bw35; Cw4; Cw5	H	2	12.5	5.0	4.3	a
IDF	A26; A2/28; Bw38; Bw18; NT	5	2	12.5	5.0	4.3	1.9
1296-S	A2; Bw51; Bw44; NT	4	3	6.2	5.0	4.8	1.9/2.1
JHH	A2; B15; Cw3	4	3	6.2	5.0	4.7	2.1
WT51 (C)	A9; B14; NT	4	3	6.2	5.0	NT	a
Mann (C)	A29; B12; Cw4	7	3	6.2	5.0	4.8	1.9
IBW9 (C)	Aw33; B14; Cw8	7	3	6.2	5.0	4.8	2.1
TF6	A1; B17; NT	7	3	6.2	5.0	4.8	a
Koz	Aw24; Aw26; Bw54; Bw40; NT	w9	3	6.2	5.0	4.8	1.9
Priess	A2; B15; Cw3	4	3	15.5/12.5*	5.0*	4.3/2.6	2.1

Approximate sizes (in kb) of polymorphic fragments, as averages from five (*Eco*RI) or three (*Taq* I) gels, were obtained from blots similar to those shown in Figs. 2 and 3. (C), from consanguineous parents; NT, not tested. a, 2.0-kb band(s) were present but gels to resolve 1.9- and 2.1-kb bands were not run. Some of the MT types are inferred; not all of the lines have been typed for MT. *Not shown in Fig. 2.

studies (unpublished data) indicate that the SB genes are arranged in the sequence $\beta\alpha\beta\alpha$ with the α and β genes having opposite orientations to one another, consistent with the above view.

What may be the reason for this increased number of genes in the HLA-D region in man? Setting aside trivial explanations, such as duplication followed by random drift, which may account for expansion or contraction of class I genes in various inbred strains of mice, it is tempting to speculate that the increased complexity of the human HLA-D region is associated with one of the distinguishing features of the species; e.g., its greater longevity or its range of habitats. These ideas are set out in more detail elsewhere (18, 19).

The DCα-chain probe, in contrast to the other α chains, detects extensive restriction fragment polymorphism in the HLA-D region, which is correlated with HLA-DR and DC types. In addition, the DCα probe reveals some variation within DR types—i.e., it identifies presumptive subtypes among individuals who have the same HLA-DR type, including variation in the DXα gene.

The combined data on the DR serology and the DNA polymorphisms detected using the DCα probe with the *Eco*RI and *Taq* I enzymes can be summarized in the form of haplotype combinations, as illustrated in Table 2. Each variable band on the Southern blots is identified by its size, and so for each homozygous cell line the combination of DR type and *Eco*RI and *Taq* I band identifies a particular haplotype. Thus, for example, the DR1 homozygous cell lines all have the EcoRI 15.5-kb and *Taq* I 2.6-kb variant bands, and so the "15.5, 2.6" haplotype. Table 2 shows the association of *Eco*RI variants with the DC or MT serology, as already discussed. The Priess cell line, which is clearly heterozygous as identified by both restriction enzymes, can be assigned a presumptive genotype 12.5, 4.3/15.5, 2.6 based on the haplotype combinations found for the other cell lines (DR3 and DR1).

Some caution is needed in interpreting these data, because it is possible that two DNA bands on a Southern blot will be found at approximately the same molecular weight position, and therefore, even for one restriction enzyme the bands of any given size may turn out to be heterogeneous. Nevertheless, it is striking that out of the many possible haplotype combinations that could be formed from the different DR types together with the *Eco*RI and *Taq* I labeled bands, only the small number shown in Table 2 have been observed, even with this set of cell lines selected to cover a wide range of HLA-DR types. The data suggest that these patterns cover the majority of haplotypes observed in the Caucasion population, from which these cell lines are derived. This restricted distribution of combinations simply reflects the expected marked linkage disequilibrium between serological and DNA detected variations.

It is intriguing that the variation found at the DNA level with the DCα probe, in contrast to the relative lack of variation found with all of the other α-chain probes, matches the high level of variation found in the mouse I-Aα chain genes. This difference in level of polymorphism is most easily explained by the action of natural selection, and it suggests that variation in the DCα chain may play an important functional role that is distinct from that for the SB and DRα chains, connected perhaps with resistance to pathogens, as has been proposed for the serologically detected variation (20).

It is well known that some HLA-DR types are significantly associated with certain diseases. The finding of variation within DR types suggests that particular subtypes may be more strongly associated with certain diseases than are the serologically defined HLA-DR types (19, 21). Previous studies have emphasized the possibility that there may be an interaction between DR3 and DR4 in determining susceptibility to insulin-dependent diabetes mellitus (22), and a similar interaction between DR4 and DR1 has been suggested (19). These interactions could reflect the association of an α chain from the haplotype carrying one of the DR types, and of a β chain from the other, to form particular $\alpha\beta$ combinations in suitable heterozygotes as also suggested by Svejgaard *et al.* (24). Since DCα is the only significantly variable α chain, the conclusion is that, more specifically, a particular DC$\alpha\beta$ combination may be involved. This idea can now be tested.

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