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Structure of crossreactive human histocompatibility antigens HLA-A28 and HLA-A2: Possible implications for the generation of HLA polymorphism

(protein sequence/genetic polymorphism/alloantigenic determinants/gene conversion)

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ABSTRACT **The primary structure of two highly crossreactive human histocompatibility antigens, HLA-A28 and HLA-A2, has been determined to 96% and 90%, respectively, of the papain-solubilized molecules. Their sequences have been compared with the sequence of HLA-B7 and with each other in order to outline the sites of diversity. The overall homology between HLA-B7 and these HLA-A antigens is 86%. A large majority of the differences are located between residues 43 and 195. Within this area, substitutions cluster in at least three segments—residues 65–80, 105–116, and 177–194. HLA-A28 and HLA-A2 show 96% homology. Most of the differences fall within segments 65–74 and 107–116. These results strongly support the suggestion that residues in these segments are integral parts of the alloantigenic determinants of HLA-A28 and HLA-A2. It is further proposed that these three clusters may constitute major, albeit not exclusive, sites of antigenic diversity in human histocompatibility antigens. The nature of the differences among HLA-B7, HLA-A28, and HLA-A2 in the first variable segment suggests that gene conversion might play some role in the generation of HLA polymorphism.**

The antigens encoded by loci *A*, *B*, and *C* of the human major histocompatibility complex (HLA) are intimately involved in the recognition of modified cells by cytolytic T lymphocytes (1–3). The most conspicuous feature of these molecules is their extensive antigenic polymorphism (4), which is believed to be essential for functional activity although its precise role remains obscure.

Extensive biochemical studies have been performed on HLA-A and HLA-B antigens (5). It is well established that they are integral membrane glycoproteins consisting of a polymorphic, MHC-encoded, heavy chain of around 44,000 daltons noncovalently bound to β_2-microglobulin, a 12,000-dalton invariant polypeptide (6, 7). Papain treatment of cell membranes solubilizes a fragment of the HLA antigen molecule (HLA_{pap}) which consists of its extracellular portion and includes the antigenic determinants (8, 9). This solubilization procedure allowed the use of conventional techniques for large-scale purification of HLA antigens (10), thus providing material for determining the complete primary structure of $HLA\text{-}B7_{pap}$ (11, 12). Analysis of this structure suggests that the heavy chain is organized in three domains of approximately 90 residues each (12). The COOH-terminal domain shows a clear homology with immunoglobulin constant domains (13), strongly supporting previous speculations that histocompatibility antigens and immunoglobulins may share a common ancestor (14, 15).

A major goal of structural studies on these antigens is determining the sites of diversity and the chemical nature of the antigenic determinants by means of sequence comparisons among various HLA molecules. It has been shown (16) that sequence diversity may be found at least through the NH_2-terminal two-thirds of the papain-solubilized heavy chain. This diversity appears to be more prominent in some short segments. Analogous results have been found in a comparison of mouse H-2K and H-2D sequences (17).

In the present report, extensive sequence data are presented for two strongly crossreactive allospecificities, HLA-A28 and HLA-A2 (18). Their comparison allows a detailed survey of molecular areas that may be responsible for alloantigenicity.

MATERIALS AND METHODS

Preparation of HLA_{pap}. Lymphoblastoid cell lines JY (A2,2; B7,7) and LB (A28,28; B40,40) were used as sources of material. Preparation of membranes, papain solubilization, and purification of both HLA-A2 and HLA-A28 were carried out as described (10). HLA heavy and light chains were separated by gel filtration in Sephadex G-75 equilibrated with 1 M acetic acid.

Purification of CNBr Fragments. Preparation of CNBr fragments of HLA-A2 has been described (16). $HLA\text{-}A28_{pap}$ heavy chain was cleaved with CNBr (19) prior to reduction and alkylation. The digest was fractionated by Sephadex G-75 gel filtration in 1 M acetic acid. Reduction and [^{14}C]carboxymethylation was subsequently carried out on one of the fractions, followed by gel filtration as above.

Peptide Purification. Tryptic and chymotryptic digestion of CNBr fragments was carried out as described (11). Peptides were purified either by microbore ion-exchange chromatography (11) or by high-pressure liquid chromatography as detailed elsewhere (20). Purification of tryptic peptides from HLA-A2 after blocking of arginine residues with cyclohexanedione (21) has been described (16). Peptides on chromatograms were detected with fluorescamine (22, 23). Radioactive cysteine peptides were detected by liquid scintillation counting.

Amino Acid Analysis. Amino acid composition of peptides was determined on a Beckman 121 M automatic amino acid analyzer after hydrolysis of the samples as described (11).

Sequence Determination. Analysis of $HLA\text{-}A2_{pap}$ heavy chain was carried out by procedures reported previously (16). Automatic Edman degradation of $HLA\text{-}A28_{pap}$ and its peptides was performed in an updated Beckman 890B or a Beckman 890C sequenator; 0.1 M Quadrol programs and Polybrene (24, 25) were used for all sequencer runs. Phenylthiohydantoin (PhCNS) derivatives were analyzed by high-pressure liquid chromatog-

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Abbreviations: HLA_{pap}, papain-solubilized HLA antigen(s); PhNCS, phenylthiohydantoin.

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raphy on a Waters Bondapak A C_{18} radial compression column (10 × 0.5 cm). A linear gradient of 40 mM NaOAc, pH 5.6/acetonitrile (18–37%) was used for 6 min followed by isocratic elution under final conditions for 6 min. PhCNS-Arg and PhCNS-His were analyzed by using a linear gradient of 40 mM NaOAc, pH 5.6/acetonitrile (40–60%) for 3 min. Elution under final conditions was subsequently maintained for 3 min more. In all cases, a flow rate of 3 ml/min was used.

RESULTS AND DISCUSSION

Sequence Analysis of HLA-A28$_{pap}$ and HLA-A2$_{pap}$ Heavy Chains. Primary structural analysis of HLA-A28$_{pap}$ heavy chain was carried out by a combination of automatic sequence determination of the intact chain, of its CNBr fragments (Fig. 1), and of tryptic and chymotryptic peptides (Fig. 2). The alignment of the sequence was done partially by overlapping peptides and partially on the basis of the sequence of HLA-B7 (12). This method was considered suitable because of the remarkable homology among different histocompatibility antigens (16, 26, 27). The lack of complete overlap analysis at this stage of the sequence determination does not allow us to exclude formally the existence of short insertions or deletions compared with the sequence of HLA-B7, although no such evidence has been found so far. The fact that both HLA-B7 and H-2K^b (27) heavy chains have exactly the same number of residues up to the papain cleavage site strongly suggests that length differences in the extracellular portion are not likely to occur. This is in contrast to the situation with immunoglobulins and may reflect a difference in the mechanisms for generation of diversity in both systems.

The data provided in Fig. 2 cover 94% of the proposed sequence of HLA-A28$_{pap}$ heavy chain, with 15 positions in which no assignment has yet been made. The single glycosylation site of the molecule at position 86 and the arrangement of disulfide bonds are identical to those of HLA-B7 (12).

The sequence of HLA-A2$_{pap}$ heavy chain is presented in Fig. 3. A sequence up to residue 167 has been reported previously

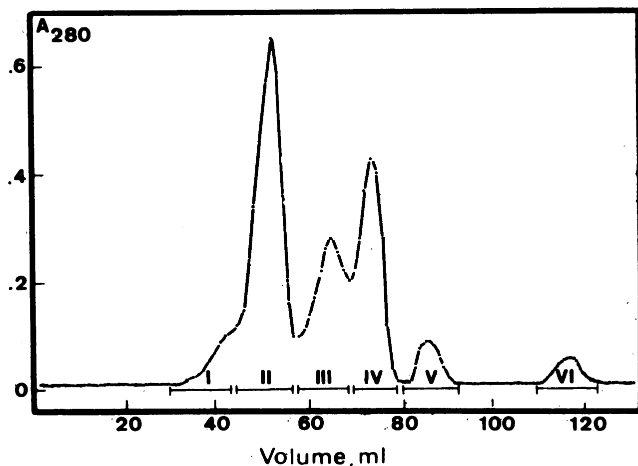

FIG. 1. Purification of HLA-A28$_{pap}$ CNBr fragments by gel-filtration chromatography on a Sephadex G-75 column (1.5 × 90 cm). Elution was carried out with 1 M acetic acid. Horizontal bars indicate the six pooled fractions. Pool I consisted mainly of undigested material. Pool II contained CNBr-4 (residues 99–138) disulfide-linked to CNBr-5 (residues 139–270). These two fragments were purified after reduction and carboxymethylation of the material contained in pool II. Pool III contained a mixture including CNBr-2 (residues 6–45) and CNBr-3 (residues 46–98) as well as a small amount of pool II material. Pools IV and V consisted of CNBr-3 and CNBr-2, respectively. CNBr-1 (residues 1–6) eluted in the included peak, pool VI.

(16). Three misassignments were detected in that sequence, at positions 142, 151, and 156. The proposed assignments for these positions are given in Fig. 3. Also, evidence for the previous assignments reported at positions 108, 114, 131, 132, and 137 was found to be insufficient. Therefore, these positions have not been assigned. The present report extends the sequence determination up to the papain cleavage point. This region of the molecule was analyzed by isolating and determining the sequence of the corresponding tryptic peptides from CNBr-5. Alignment of these peptides was based on the sequence of HLA-B7, as discussed above for HLA-A28. Thus, the proposed sequence includes 90% of HLA-A2$_{pap}$ heavy chain residues. Both the glycosylation site and the arrangement of disulfide bridges are the same as in HLA-B7 and HLA-A28. These data add to the existing evidence concerning the strong conservation of such structural features in histocompatibility antigens (26, 28). In contrast to the mouse system (26, 29), multiple glycosylation sites were not found in HLA-A28 or HLA-A2.

Comparison of HLA-A28$_{pap}$ and HLA-A2$_{pap}$ Sequences with HLA-B7$_{pap}$ Sequence. The available sequences of HLA-A28 and HLA-A2 were aligned with the complete sequence of HLA-B7$_{pap}$ (Fig. 4): 253 positions were compared between HLA-B7 and HLA-A28 and 242 residues, between HLA-B7 and HLA-A2. Thus, these comparisons involve 94% and 90% of the sequences respectively. Because products of different loci are being considered, it is clear that an outline of sequence diversity between HLA-A28 or HLA-A2 and HLA-B7 may reflect a number of different determinants. HLA-B7$_{pap}$ differs from both HLA-A28$_{pap}$ and HLA-A2$_{pap}$ in 35 of 253 and 242 positions compared, respectively. This means an overall homology of 86% between HLA-B7$_{pap}$ and either of the two HLA-A specificities.

The distribution of differences is not uniform, most of them being located between residues 43 and 195 (Fig. 4). Within this region, conserved segments alternate with areas in which substitutions accumulate. At least three clusters of differences may be identified: positions 65–80, 105–116, and 177–194. Sequence homology within these segments decreases to 31%, 50%, and 56%, respectively. Strikingly, the positions of the last two HLA diversity segments in relation to the cysteine residues of the first disulfide bond (Cys-101–Cys-164) are reminiscent of the location of the first and third hypervariable regions of immunoglobulin heavy chains (30). A fourth, less-defined, cluster spans residues 138–156, the homology in this segment being 63%. As much as 74% of the substitutions fall within these four segments which together encompass 65 residues or only 25% of the molecule. Their importance as putative sites for alloantigenic determinants is suggested by the fact that essentially all residue substitutions that have been determined in mouse H-2K^b mutants fall within these clusters (31).

The most conspicuous area of diversity is the segment 65–80. It also shows a comparable degree of variability in murine histocompatibility antigens (17) and its implication in the structure of alloantigenic determinants has been suggested (16, 17). A remarkable accumulation of differences is found between HLA-B7 and H-2K^b in the region around residues 177–194, suggesting that this area could also constitute an important diversity site in H-2 antigens. Genetic analysis of histocompatibility genes (32–34) indicates that histocompatibility antigens are encoded by exons that correlate with protein domains as suggested by internal sequence homology in HLA-B7 (12). Thus, a splicing point joining the coding exons for the second and third domains of the molecule should correspond to residue 183 (32), which lies within the cluster of variability at residues 177–194. This segment may be a rather extended interdomain region and conceivably could tolerate diversity without disturbing the overall structure.

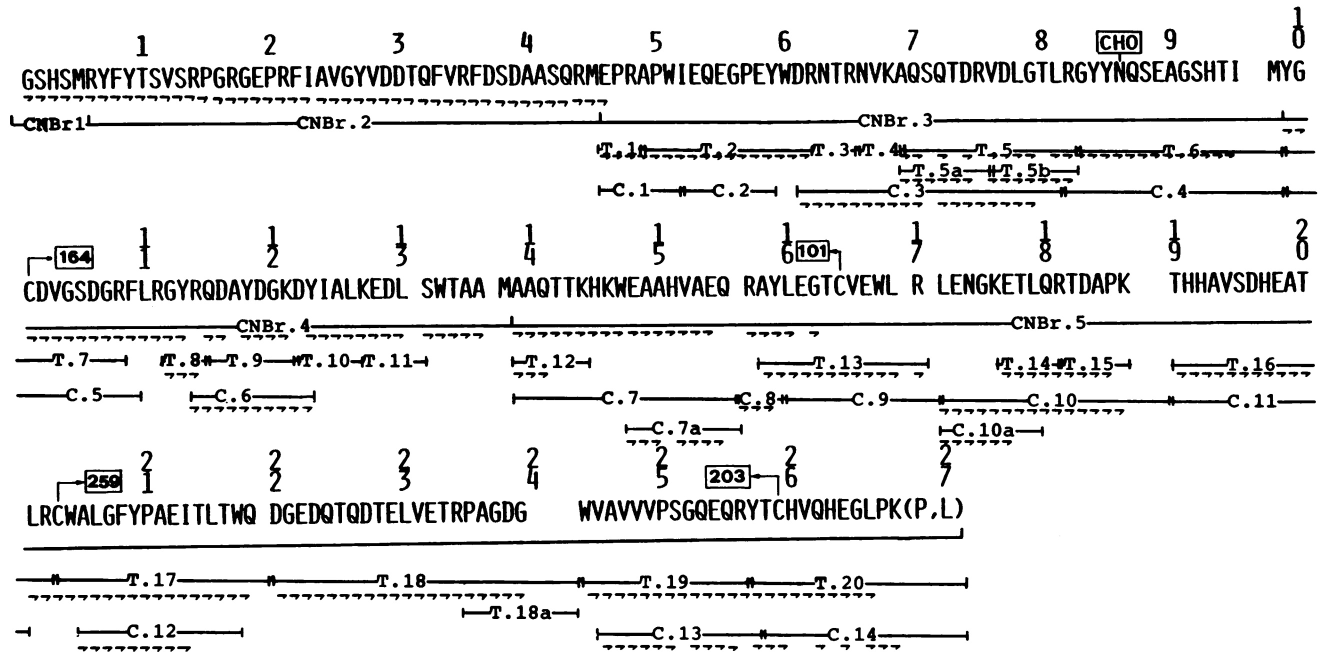

FIG. 2. Primary structure of HLA-A28$_{pap}$ heavy chain. Residue numbering corresponds to that of HLA-B7 (12). Blanks are nonassigned positions. T, tryptic peptides; C, chymotryptic peptides; arrows, residues identified after automatic Edman degradation. The following one-letter code for amino acids is used: A, Ala; B, Asx; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; Z, Glx.

Only four substitutions are found in the NH_2-terminal 64 residues (Fig. 4), giving a homology of 94% in this region. Similarly, in the COOH-terminal portion, residues 195–270 which include the second disulfide bridge of the molecule, only three differences have been detected (positions 239, 245, and 253). Thus, the homology in this region is 96%. Although it is likely that these regions may not contain major sites of diversity (16, 35), their contribution to some of the antigenic polymorphism should not be disregarded. For example, the H-2K^b mutant bm8 has been shown to differ from the parental H-2K^b only at position 23 (31); thus, this position may be important in triggering graft rejection. In addition, a substitution involving a charge difference has been located in the tryptic peptide spanning residues 36–44 by comparing two serologically identical HLA-A2 molecules which are distinguishable in an *in vitro* cellular cytotoxicity assay (M. Krangel, personal communication). In the COOH-terminal region, substitutions at positions 239 and 253 also involve charge differences. If the COOH-terminal domain of the molecule is folded like an immunoglobulin constant domain (13), those residues would be located in turning loops of

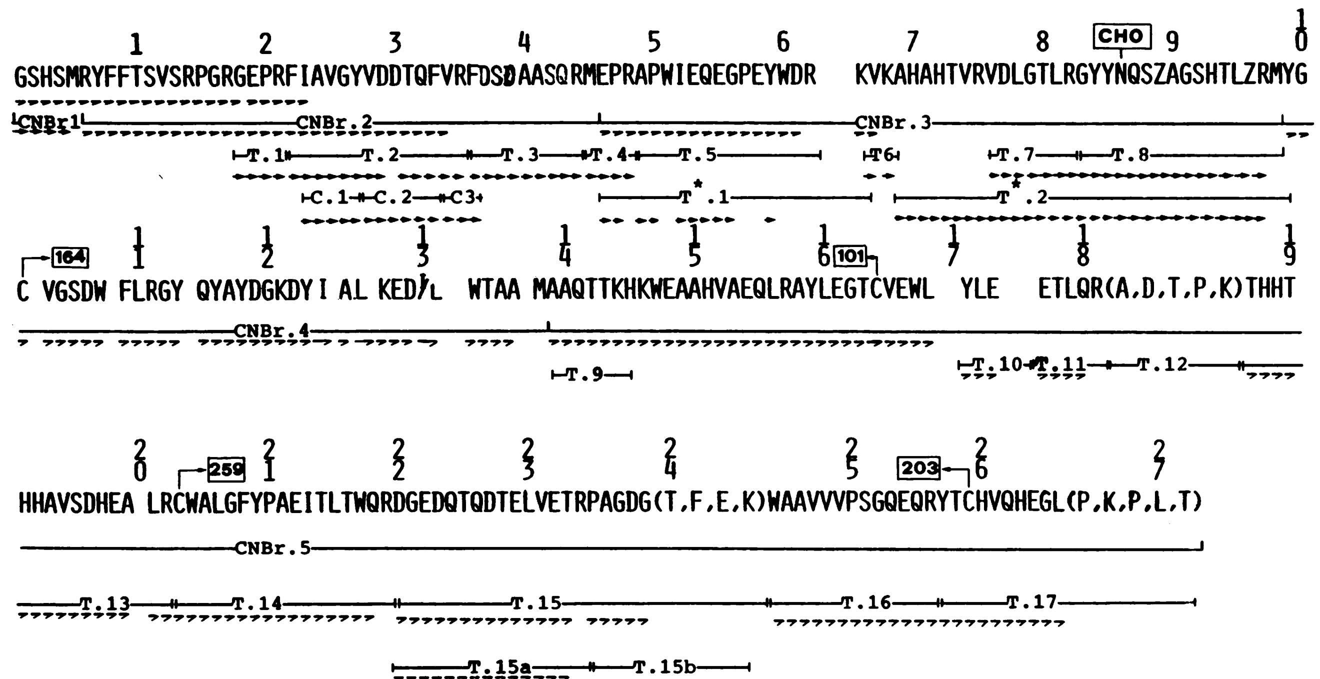

FIG. 3. Primary structure of HLA-A2$_{pap}$ heavy chain. Residue numbering and symbols are as in Fig. 2. Additional symbols: T*, tryptic peptides obtained after Arg blockage; ⇀, positions determined by automated procedure; →, positions determined manually.

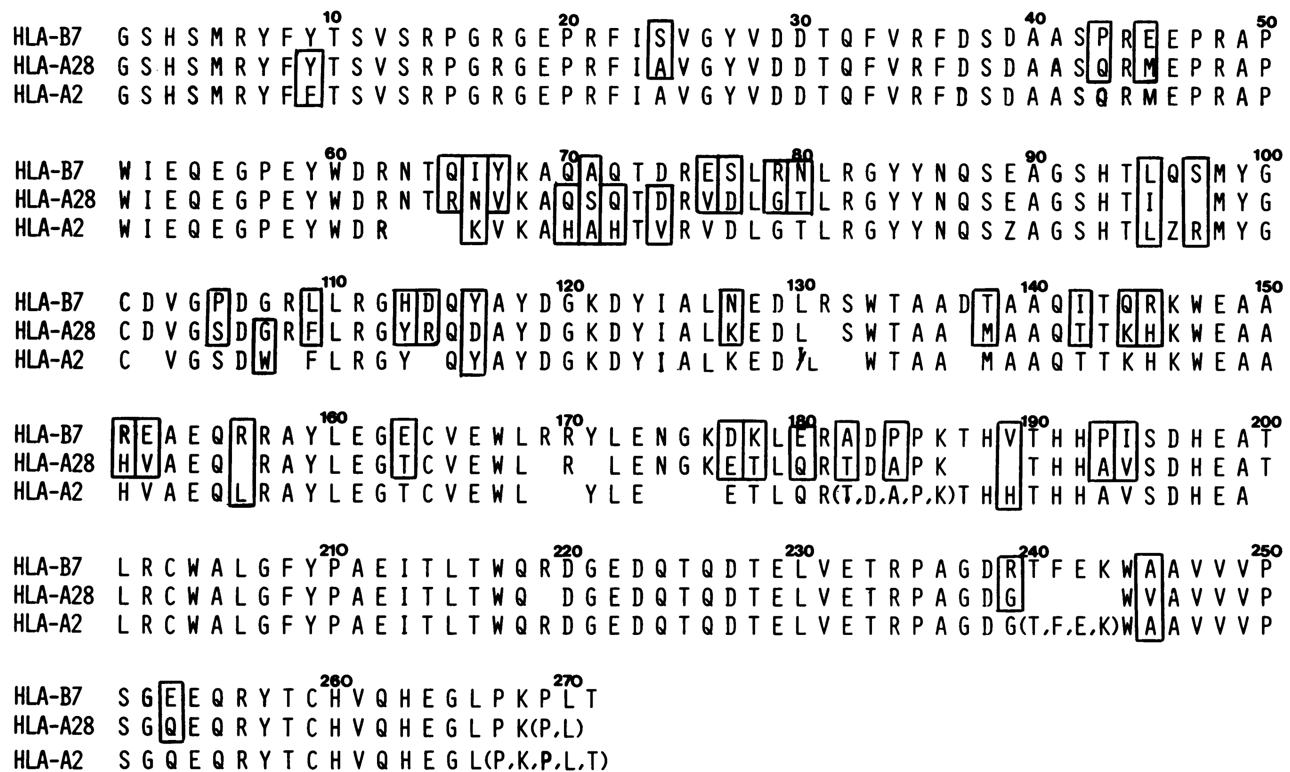

FIG. 4. Comparison of the primary structures of HLA-A28$_{pap}$, HLA-A2$_{pap}$, and HLA-B7$_{pap}$ heavy chains. HLA-B7 sequence is taken from ref. 12. Boxed residues are those that are different among the proteins being compared. Blanks correspond to nonassigned positions.

the β-pleated sheets and conceivably could contribute to antigenic determinants.

Comparison Between HLA-A28$_{pap}$ and HLA-A2$_{pap}$ Heavy Chain Sequences. Primary structural analysis of HLA-A28 and HLA-A2 was undertaken on the basis that their antigenic similarity (18) should reflect a correspondingly high sequence homology. Hence, the location of amino acid residue differences would allow a more accurate mapping of alloantigenic sites. In the alignment of both sequences (Fig. 4), 235 of 270 positions (87%) are compared. The overall homology is 96% with only 10 residue differences (positions 9, 66, 70, 71, 72, 74, 95, 107, 116, and 245). Seven of them lie within two of the clusters of diversity discussed above—i.e., residues 65–80 and 105–116. The three substitutions that are located outside (positions 9, 95, and 245) are quite conservative and thus it is unlikely that they play a significant role in the alloantigenicity of these molecules. In contrast, those differences falling within the clusters show a greater chemical disparity. Two substitutions in the first segment (residues 66 and 74) and one in the second (residue 116) involve a charge difference. In addition, the Gly/Trp interchange at position 107 is also nonconservative. Thus, at least two of the segments defined above as areas of diversity on the basis of sequence comparisons may constitute a major part of the alloantigenic determinant(s) that differentiate HLA-A28 and HLA-A2 histocompatibility antigens. It is possible that additional differences may still emerge, especially in the region 173–189 where preliminary evidence suggests that some substitutions will be encountered upon completion of HLA-A28 and HLA-A2 sequence determinations in this area.

Gene Conversion as a Possible Mechanism for Generation of HLA Polymorphism. The nature of the differences among HLA-A28, HLA-A2, and HLA-B7 is such that only 1 of 235 positions available for comparison is different in all three molecules (residue 66). In 5 of 10 detected differences between HLA-A28 and HLA-A2, the corresponding residue in HLA-B7 is identical to HLA-A28 (positions 9, 70, 72, 74, and 107); in 4 positions HLA-B7 and HLA-A2 are identical (residues 71, 95, 116, and 245). Although it is conceivable that structural or functional constraints could restrict diversity at many positions, the overall pattern of variability is most easily explained as a direct consequence of a recent diversification of the corresponding genes, so that only a limited number of point mutations accumulated in any of them. It is particularly noteworthy that 4 of 10 differences between HLA-A2 and HLA-A28 are clustered in the segment spanning residues 70 to 74. However, HLA-B7 differs only in one position from HLA-A28 in the sequence of this segment (Fig. 4). The limited number of substitutions involved and the fact that each one may be accounted for by a single base change in the corresponding codons does not allow one to eliminate the possibility that this variability has been generated by a mechanism of point mutations.

However, the alternative possibility that gene conversion affecting short segments of highly variable areas was responsible for the observed pattern must also be considered. Gene conversion is a nonreciprocal recombinational event by which a particular segment of a gene becomes identical to the corresponding portion of another related but nonidentical gene (36, 37). The process has been proposed to be an advantageous way of maintaining sequence homogeneity in gene families, such as those of immunoglobulins (37). It has been invoked to explain the occurrence of sequential homologies between nonallelic genes in areas in which alleles are significantly nonhomologous (38, 39), a pattern analogous to that found in HLA antigens except for the fact that a smaller segment is involved in this case. Thus, if gene conversion occurs between members of a gene family within a highly homologous region, sequence homogeneity will be enhanced. However, if it occurs within diversity regions of these genes affecting only a portion of these regions,

polymorphism will be increased because the polymorphic stretch of the converted gene will be different from both the parental and the converting genes.

Indirect evidence for the involvement of gene conversion in the evolution of histocompatibility antigens has also been provided by the recent findings by Evans *et al.* (33) concerning the structural relationships of H-2L^d antigen with an H-2K^b mutant K^{bm1}. This mutant differs from the parental H-2K^b molecule in two contiguous amino acid residue substitutions which would require a minimum of two base changes in each of the corresponding codons (31). This fact makes their generation by point mutations unlikely. However, these residues in H-2K^{bm1} are identical to the corresponding residues in H-2L^d.

The data presented in this paper have the limitation that the segments involved in putative gene conversion events are very short. Therefore, it is unlikely that they would significantly alter the apparent diversification rate of the genes, as expected from point mutation mechanisms. In the absence of such detectable alterations, conclusions regarding the possible role of gene conversion in the generation of HLA polymorphism have to be regarded with caution.

Location of Antigenic Determinants. At least two monoclonal antibodies have been found that are able to split HLA-A28 and HLA-A2 while still reacting significantly with some HLA-B antigens (40, 41). One of these antibodies, MB40.1, reacts strongly with both HLA-B7 and HLA-A28 and more weakly with HLA-A2 (41). It is likely that some of those residues that are identical in both HLA-A28 and HLA-B7 but different in HLA-A2 may be primarily involved in the antigenic determinant recognized by this antibody. Of five differences only two (residues 74 and 107) are clearly nonconservative and only one (residue 74) involves a charge difference. Thus, short of having available the complete sequences of HLA-A28 and HLA-A2, it may be tentatively suggested that Asp-74 in HLA-A28 and HLA-B7 (Fig. 4) constitutes an important portion of the antigenic determinant recognized by MB40.1 antibody.

The data discussed above demonstrate that it may be possible to undertake the mapping of antigenic determinants by correlating primary structural analysis of histocompatibility antigens with HLA serologic features. Similarly, it is expected that the complex reactivity patterns and antigenic relationships revealed by anti-HLA monoclonal antibodies (42) will progressively be interpreted in molecular terms by comparative structural analysis of appropriate specificities.

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