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Allelic diversification at the class II *DQB* locus of the mammalian major histocompatibility complex

(polymerase chain reaction/evolution/selection/polymorphism)

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ABSTRACT **The allelic diversity at *HLA* class II loci either arose after the divergence of hominoid lineages or, alternatively, the polymorphism was present before speciation and has been maintained by selection. Here, we report the use of oligonucleotide primers to amplify, by the polymerase chain reaction, and sequence the polymorphic second exon of the *DQB* locus from 11 species, spanning more than 40 million years of mammalian evolution. Phylogenetic analysis reveals that of the four human *DQB* allelic types (*DQB1–B4*), three (*DQB1–3*) were found in chimpanzee and gorilla and two (*DQB3* and *-4*) were identified in the rhesus monkey, suggesting that some of these types are 5–20 million years old. The ratio of replacement to silent substitutions was calculated between members of the same allelic type from different species. These results suggest that the evolution of the *DQB3* allelic type is more constrained than that of the *DQB1* allelic type; both evolve more slowly than the *DXB* locus, a linked but presumably nonexpressed locus. Further, the clustering of allelic subtypes by species in the phylogenetic tree indicates that allelic diversification has occurred subsequent to the divergence of hominoids. Finally, some haplotype combinations of *DQA* and *DQB* alleles are common to several hominoid species and may have been maintained for at least 5 million years.**

The *HLA-D* or class II region consists of three subregions, *HLA-DR*, *-DQ*, and *-DP*, each of which encodes an α and at least one β glycoprotein chain (1, 2). These chains form a highly polymorphic integral membrane protein that binds peptide fragments derived from processed antigens. The peptide fragment is thought to be located within a putative antigen-binding cleft formed by two α-helices and a β-pleated sheet in the N-terminal outer domain of the heterodimer (3). The recognition of this peptide–class II molecule complex by the T-cell receptor leads to T-lymphocyte activation. Virtually all of the nucleotide sequence polymorphism of the class II loci is localized to the second exon that encodes both the α-helix and the β-pleated sheet (1–5).

The origin and maintenance of the extensive polymorphism at the HLA class II loci have been the subject of considerable controversy. It has been suggested that recent mutations and/or recombination and gene conversion events, followed by selection for variability, gave rise to the observed polymorphism (6). An alternative explanation has been that the alleles in the human population were present in the ancestral species prior to the divergence of the hominoid lineages (7) and have been maintained either by selective mechanisms such as overdominance (8) or by negative assortative mating (9). We have shown (10) that most of the alleles at the *HLA-DQA* locus were present in the ancestral species that gave rise to the human, chimpanzee, and gorilla (hominoid) lineages and are, therefore, at least 5 million years old. The results of analysis of the *DQA* locus are consistent with the model of ancient allelic diversity, as are the results of other studies on the major histocompatibility complex polymorphism (11–16). Since the class II loci encoding α chains are less polymorphic than the class II loci encoding β chains, the evolutionary history of the polymorphism at the two types of loci may be different. To investigate the evolution of *DQB* polymorphism, we have determined the nucleotide sequence of the second exon by enzymatic amplification of samples from 11 mammalian species and subjected them to phylogenetic analysis.

MATERIALS AND METHODS

DNA samples from 30 humans of varied ethnical origin (Caucasian, Black, or Asian) (4), 13 chimpanzees (*Pan troglodytes*), 4 pygmy chimpanzees (*Pan paniscus*), 6 gorillas (*Gorilla gorilla*), 1 baboon (*Papio leucophaeus*), 2 rhesus monkeys (*Macaca mulatta*), 2 langurs (*Presbytis entellus*), 1 capuchin monkey (*Cebus capuchinus*), 2 horses (*Equus equus*), 1 sheep (*Ovis ovis*), and 1 beluga (*Delphinapterus leucas*) were subjected to 30 cycles of the polymerase chain reaction (PCR) (17–19) using oligonucleotide primers GH28 and GH29 and amplification conditions as described (4). These primers are complementary to regions within the second exon of the *HLA-DQB* locus and have a *Bam*HI (GH28) or a *Pst* I (GH29) restriction enzyme site attached to their 5′ ends, facilitating cloning of the PCR products. Due to sequence homology, these primers coamplify the *DXB* locus, a presumably nonexpressed and linked locus. Amplified DNA fragments were digested with *Bam*HI and *Pst* I and ligated into M13mp18. Phages containing PCR products were identified by plaque hybridization with an oligonucleotide made to an invariant region of the second exon and sequenced by the chain-termination method (20). Each allelic sequence was confirmed by analysis of at least two clones.

For the haplotype analysis, amplified DNA samples were typed with a set of 13 horseradish peroxidase-labeled oligonucleotides (T. L. Bugawan and H.A.E., unpublished data), diagnostic for the *HDQB1–B4* allelic types. The polymorphic region *D* of the second exon (see Fig. 4), which is outside the oligonucleotides GH28 and GH29, was analyzed using GH28 and UG74 (5′-ATCCCGCGGTACGCCACCT-3′), a PCR primer that only amplifies the *DQB1* allelic type.

Phylogenetic trees for the sequences were constructed by (*i*) maximum parsimony analysis (21, 22) using the computer programs PAUP (David Swofford, Illinois Natural History Survey, Champaign, IL), PHYLIP (Joe Felsenstein, University of Washington, Seattle), and MACCLADE (Wayne Maddison, Harvard University) and (*ii*) distance analysis using the neighbor-joining method (23). The parsimony analyses were based on (*i*) phylogenetically informative amino acid positions, (*ii*) phylogenetically informative nucleotide posi-

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Abbreviation: PCR, polymerase chain reaction.

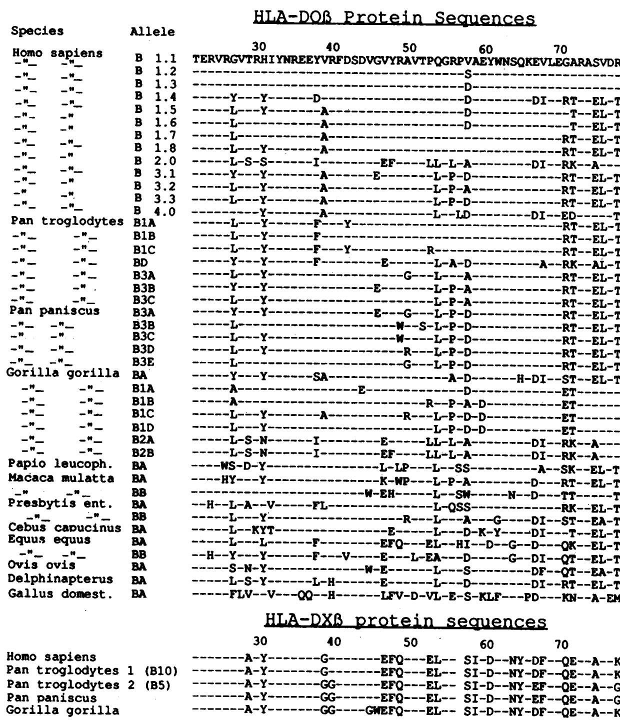

FIG. 1. Alignment of the amino acid sequences of the second exon (codons 21–77) of the *HLA-DQB* (*Upper*) and *HLA-DXB* (*Lower*) locus from 11 mammalian species and one bird (27). The primer sequences are not included in the alignment. The single-letter amino acid code is used.

tions, and (*iii*) phylogenetically informative third positions of the codons of the nucleotide sequences. The actual and potential number of replacement and silent substitutions between sequences were estimated according to Perler *et al.* (24) and deviations were tested for statistical significance by χ^2 analysis.

In previous work on *HLA* class II sequence polymorphism, this laboratory has used the locus nomenclature DQα and DXα and designated the alleles at the DQα locus A1–A4 (4, 10). A system of nomenclature for the class II loci has been introduced in which DQα is designated *DQA1* and DXα is *DQA2* (25). Similarly, the DQβ locus have been designated *DQB1* and DXβ is *DQB2* (25). To avoid confusion between the locus and allele description, we have in this paper denoted the DQβ locus *DQB* and the DXβ locus *DXB* but retained our previous nomenclature for allelic variants (*DQB1–4*) (4). The human sequence defined alleles *DQB1.1*, *1.2*, and *1.3* (B1a group) correspond to the serologic specificities DQw5 and the alleles *B1.4*, *1.5*, *1.6*, *1.7*, and *1.8* (B1b group) correspond to the DQw6 specificity (4).

RESULTS

The oligonucleotide primers GH28 and GH29, based on regions of the second exon that are conserved among all human *HLA-DQB* alleles, were capable of priming class II β-chain sequences in species that diverged from the primate lineage 40–60 million years ago. An alignment of 48 amino acid sequences of the second exon from the *DQB* and *DXB* loci [including 33 sequences not previously reported, 13 human alleles (4, 26), and a sequence from domestic chicken

FIG. 2. Alignment of the corresponding nucleotide sequences of the second exon of the *HLA-DQB* and *HLA-DXB* locus from 11 mammalian species and one bird (27), arranged by species.

(27) used as an outgroup in the phylogenetic analysis] is shown in Fig. 1, and the corresponding alignment of DNA sequences is shown in Fig. 2. The *DQB* locus was polymorphic in all species where several individuals were analyzed; both chimpanzee species (*Pan troglodytes* and *Pan paniscus*) and the gorilla also contain sequences homologous to the closely linked but unexpressed *DXB* locus, which has a very low level of polymorphism in humans [a silent G-C at codon 25 and a GAG (glutamic acid)-GGG (glycine) at codon 35; H.A.E., unpublished results].

The 13 human *DQB* alleles are derived from the four major allelic types *DQB1* (with eight subtypes), *DQB2*, *DQB3* (with three subtypes), and *DQB4*, corresponding to the serologic specificities DQw1, DQw2, DQw3, and DQw4, respectively (4, 26). Phylogenetic analysis of the amino acid sequences from all the species, using estimates of genetic distance and the neighbor-joining method (23), shows that most of the *Pan* (of both species) and *Gorilla* sequences cluster with a particular allelic type found in the contemporary human population (Fig. 3*A*). Similar results were obtained using a parsimony analysis of the amino acid sequences (Fig. 3*B*), indicating that the pattern of sequence relationships is independent of the method used for phylogenetic reconstruction. Parsimony analysis using the nucleotide sequences also resulted in very similar topology (data not shown). Thus, a given human allele (e.g., *DQB3.1*) is more similar to its counterpart in *Pan* (e.g., *PT3B* and *PPB3A*) than it is to the human *DQB1*, *DQB2*, or *DQB4* allelic types. The pattern of clustering of sequences by allelic types rather than by species indicates that the *DQB1–B3* allelic types were present in the ancestral species that gave rise to the various hominoid lineages and are, therefore, at least 5 million years old (28, 29). To ascertain that the branching order in the tree was not due to convergent evolution of the protein sequences (30), parsimony analysis was also performed using the polymorphisms at third positions of the codons of the nucleotide sequences. Several parsimony trees with equal length were obtained; the consensus tree clustered sequences by allelic type rather than by species (data not shown). Thus, the similarity of allelic types between species is due to common ancestry and not convergent evolution of the amino acid sequences.

Different subsets of the contemporary human allelic types were found in the nonhuman hominoids. In *Pan troglodytes*, *DQB1*, *DQB3*, and a *DQB2*-related sequences were found, whereas in *Pan paniscus* only *DQB3* sequences were found. In the *Gorilla*, both *DQB1* and *DQB2* sequences were found and, in addition, two sequences that showed similarity to a *DQB1* type by phylogenetic analysis. The *Macaca 1* sequence clusters with the *DQB3* group, as do the sequences from *Delphinapterus* and *Presbytis 1* with the *DQB2* allelic type, indicating that these allelic types may be more than 20 million years old (31, 32).

The trees for DQB protein sequences also indicate that divergence has occurred within the ancient allelic types subsequent to speciation (Fig. 3). Within the *DQB1* clade, the sequences cluster by species both in the neighbor-joining tree and the parsimony tree, indicative of their recent origin. In the *DQB3* clade, the sequences also appear to form species groups, with the exception of two sequences (Fig. 3). The consensus parsimony tree based on nucleotide sequences also cluster the *DQB1* sequences by species. Thus, both types of phylogenetic analyses indicate that subtypic diversification has occurred after speciation.

The extent of evolutionary constraint on the amino acid sequence encoded by a particular *allelic type* was estimated from the ratio of replacement to silent substitutions in pairwise comparisons of sequences of the same *DQB* allelic type in different hominoids. A lower ratio than the 3:1 expected for genes under weak functional constraints would imply that the protein is constrained from rapid evolution, while a higher

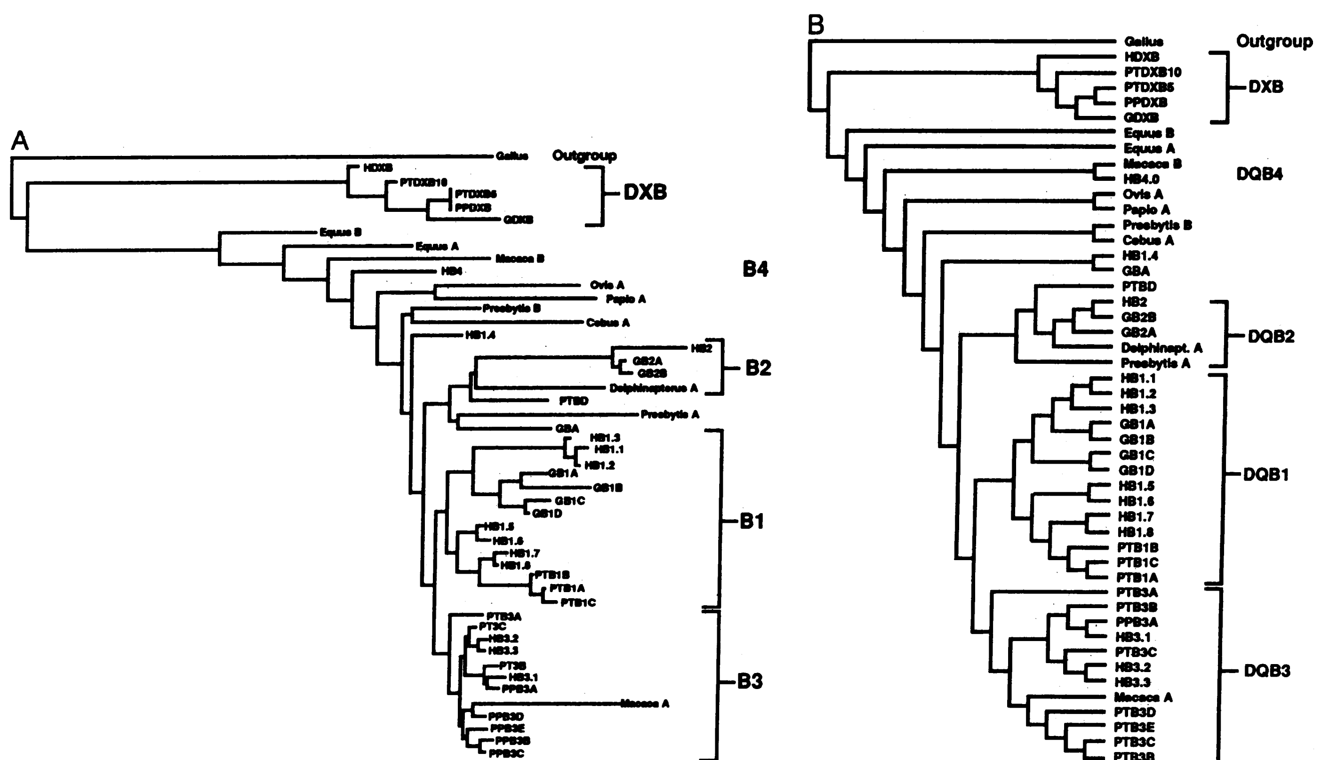

FIG. 3. (*A*) Phylogenetic tree based on the genetic distance between the amino acid sequences. The tree was generated by the neighbor-joining method (23) and the bird sequence was used as an outgroup. Branch lengths are proportional to the amount of changes. (*B*) Phylogenetic tree constructed by maximum parsimony from the phylogenetically informative positions of the amino acid sequences. The parsimony network was transferred into a tree by using the bird sequence as an outgroup. The tree shown is the consensus tree of several equally parsimonious trees that differ by rearrangements within the B3 cluster (see text). Branch lengths are not proportional to the amount of change.

ratio would indicate selection for variability. The average ratio for the *DXB* locus among hominoids is 3/0.78 = 3.84 (Table 1), consistent with the expectation for an unexpressed (hence "neutral") locus. By this measure the *DQB1* (9.15/4.15 = 2.2) and the *DQB3* (4.31/2.85 = 1.5) allelic types appears to evolve more slowly than the *DXB* locus (Table 1). A statistical comparison of actual and potential numbers of silent and replacement differences shows that the amino acid sequence of DQB3 is significantly constrained (Table 1).

The combination of *DQA* and *DQB* alleles on 46 nonhuman hominoid chromosomes was studied by nucleotide sequence analysis and oligonucleotide typing of amplified DNA, and 36 haplotypic combinations were determined from homozygous individuals (Table 2). Three (*DQA1–DQB1*, *DQA3–DQB3*, *DQA4–DQB3*) of the five different haplotypes found appear to be conserved in at least three of the four hominoid species, while two haplotypes are unique to nonhuman hominoids (*DQA3–DQB1*, *DQA4–DQB1*). In general, strong linkage disequilibrium between the *DQA1* and *DQB1* alleles was observed among the nonhuman hominoids, suggesting an evolutionary maintenance of this haplotype.

DISCUSSION

The evolution of allelic polymorphism at the second exon of the class II *DQB* locus was studied in a series of mammals by using PCR amplification. The four major allelic types at the *DQB* locus in the contemporary human population have persisted for at least 5 million years and some of the types have persisted for even longer periods of time (at least 20 million years), although representatives of all four types were not detected in all species. A similar estimate of the age of the allelic types was calculated for the *DQA* locus (10). This observation is hard to reconcile with the notion of rapid evolutionary change at these loci and favors the view that many of the allelic types are ancient and maintained by some form of selection. Several sequences derived from *Pan*, *Gorilla*, and the other species do not cluster with any particular allelic type; these either represent allelic types not transferred between species or sequences that have evolved beyond recognition as members of the *DQB1–B4* types. No counterpart to the human *DQB4* was found in any of the other hominoids; this allelic type may be very rare in these species and may have gone undetected in this survey of hominoid alleles. Alternatively, the *DQB4* allelic type could have arisen within the human lineage or been present in an ancestral species and passed on only to the human lineage. The similarity of the human *DQB4* allele to an Old World monkey sequence in the parsimony tree (*Macaca 3*, Fig. 3) suggests that the latter hypothesis may be more likely than the proposal of post-speciation origin. The presence of *DXB* sequences in the hominoids but not in the Old World monkeys suggests that this locus may have arisen from a gene duplication after the split of hominoids from Old World monkeys.

The allelic subtypes, however, seem to have arisen primarily after divergence of hominoid lineages. This is evident from the clustering of *DQB1* subtypes by species in the phylogenetic tree (Fig. 3) and also indicated for the *DQB3* and *DQB2*, and to some extent for the *DQA4* subtypes (10). Thus, in the evolution of the *DQB* polymorphism, the two models for major histocompatibility complex evolution mentioned in the introduction are not mutually exclusive explanations; the major allelic types have been maintained over evolutionary time periods but species-specific subtypes have been generated more recently.

The available sequence data suggest that both point mutation and recombination/gene conversion contribute to the generation of allelic diversity. Many of the allelic subtypes

Table 2. Combination of alleles at the *HLA-DQA* and *-DQB* loci in primates

Species	*n*	Genotype: *DQA*	Genotype: *DQB*
Homo		*1.1*	*1.1* (Dw1), *1.3* (Dw9)
		1.2	*1.2* (AZH), *1.3* (Dw9), *1.5* (Dw2), *1.7* (Dw19)
		1.3	*1.4* (Dw12), *1.6* (Dw18)
		2	*2*, *3.3*
		3	*3.1*, *3.2*, *3.3*, *2* (Black DR7), *4* (Japanese DR4)
		4	*2*, *3.1*, *4*
Pan troglodytes			
	3	*1/1* (s, o)	*1/1* (s, o)
	2	*3/3* (s)	*3/3* (s, o)
	3	*3/3* (s, o)	*1/1* (s, o)
	1	*4/4* (s)	*1/1* (s, o)
	1	*4/4* (s)	*3/3* (s)
	1	*3/3* (o)	*1/3* (s)
	2	*4/4* (o)	*1/3* (o)
Pan paniscus			
	1	*4/4* (o)	*3/3* (s, o)
	1	*3/4* (o)	*3/3* (s)
	2	*3/4* (o)	*3/1* (s)
Gorilla gorilla			
	1	*4/4* (o)	*1/1* (s, o)
	2	*1/4* (s)	*1/1* (s, o)
	2	*1/4* (s)	*2/1* (s)
	1	*1/3* (s)	*2/1* (s)

Genotypes of nonhuman hominoids were determined by sequencing of cloned PCR products (s) or by typing with oligonucleotides (o). *n* is the number of haplotypes examined.

Table 1. Substitutions among hominoid *DQB* and *DXB* alleles

	Substitutions, no.															
	DQB1				*DQB2*				*DQB3*				*DXB*			
	s		r		s		r		s		r		s		r	
Comparison	a	p	a	p	a	p	a	p	a	p	a	p	a	p	a	p
Homo vs. *Pan t.*	3.14	63.3	7.7	141.2	—	—	—	—	3.83	64.05	4.39	141.9*	0.5	61	2	145.7
Homo vs. *Pan p.*	—	—	—	—	—	—	—	—	3.37	63.56	4.43	142.5*	0	61	4	144
Homo vs. *Gorilla*	4.86	63.7	9.36	141.5	0	66	1.5	144	—	—	—	—	1	60.5	3	146.5
Pan p. vs. *Pan t.*	6	63.7	13.67	141.7	—	—	—	—	1.73	63.50	4.13	142.1	0.5	60.5	2	144
Pan t. vs. *Gorilla*	—	—	—	—	—	—	—	—	—	—	—	—	1.5	60.7	3.5	146
Pan p. vs. *Gorilla*	—	—	—	—	—	—	—	—	—	—	—	—	1	61	5	142
Average	4.15	63.53	9.15	141.6	0	66	1.5	144	2.85	63.65	4.31	142.2*	0.78	60.8	3	144.9

Pan t., *Pan troglodytes*; *Pan p.*, *Pan paniscus*. Actual (a) and potential (p) numbers of nonsynonymous (r) and synonymous (s) substitutions among hominoid *DQB* and *DXB* alleles, estimated according to Perler *et al.* (24). Average for all pairwise comparisons. *$P < 0.01$ for deviations of the actual ratio of replacement to silent changes from the potential ratio.

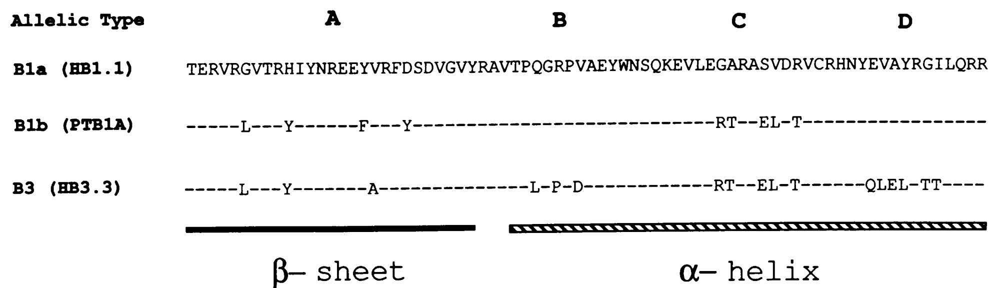

FIG. 4. Allelic types at the *DQB* locus. The capital letters indicate the polymorphic regions (*A*–*D*) of the second exon corresponding to the putative β-strands and α-helices of the class II structural model (3). Polymorphic region *D* was analyzed in the humans by cloning and sequencing and in the nonhuman primates by using a second set of PCR primers and oligonucleotide typing (see text).

differ by single amino acids that could have been generated by single point mutations. By contrast, in some cases a given allelic type could have been generated by recombination between two other contemporary allelic types. For example, the *DQB1b* allelic type (represented by *HDQB1.4–1.8*) could have arisen by several events of segmental transfer between the *DQB1a* (*HDQB1.1–1.3*) and *DQB3* types (Fig. 4). The presence of the *DQB1b* allelic type in both chimpanzee and human suggests that these putative intraexon recombinational events between sequences encoding the β-sheet and α-helix must have taken place before speciation (>5 million years ago).

Moreover, the maintenance of allelic types over evolutionary periods of time is inconsistent with the proposal that the polymorphism is neutral. For alleles at *DQB* and *DQA* (10) to be maintained for 5–20 million years by stochastic forces only, we have to assume effective population sizes that are unrealistically large for these primates (10). The ratio of replacement to silent changes indicates that most *DQB* allelic types, like the *DQA* allelic types (10), are evolving slower or at the same rate as nonexpressed *HLA* loci, inconsistent with the view of rapid generation of variation. Instead, the protein sequence of the allelic types are conserved, possibly by some form of balancing selection (8).

The haplotypic combination of alleles at the *DQA* and *DQB* loci in humans is restricted, and in some cases a specific *DQA* allelic type (e.g., *A1*) is found only in combination with a specific *DQB* allelic type (e.g., *B1*) (Table 2). This is particularly striking for the very restricted haplotypic combinations of *DQA1* subtypes (*A1.1–1.3*) and *DQB1* subtypes (*B1.1–1.8*) (Table 2). Unlike the haplotypic association in humans, in the nonhuman hominoids, *DQB1* alleles are found on haplotypes that carry *DQA* alleles other than *DQA1* (Table 2). However, only *DQB1* alleles are found on haplotypes with *DQA1*, indicating that this haplotype combination may have been maintained for more than 5 million years. Even though the *DQA* and *DQB* loci are tightly linked [e.g., within 12 kilobases (33)], the divergence time between human and chimpanzee is sufficient to uncouple linked genes at this distance, given no selection and no molecular suppression of recombination. We have previously suggested that the preferential association of certain β chains with specific α chains may account for the differing rates of evolution for different α-chain alleles (10). This putative constraint on α–β chain pairing may also explain the evolutionary maintenance of specific haplotypic combinations (e.g., *A1–B1*) of α- and β-chain alleles.

In summary, the four allelic types (*B1–B4*) at the *DQB* locus appears to be ancient (>5 million years old), although not all types were found in each hominoid species. The diversity within these ancient allelic types, however, have been generated after speciation. The evolutionary maintenance of allelic types is presumably the result of selection for preservation of functional diversity, while the persistence of certain haplotypic combinations of *DQA* and *DQB* alleles may reflect constraints on α–β chain association.

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