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Mutations and selection in the generation of class II histocompatibility antigen polymorphism

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A comparison of seven human DR and DC class II histocompatibility antigen β-chain amino acid sequences indicates that the allelic variation is of comparable magnitude within the DR and DC β-chain genes. Silent and replacement nucleotide substitutions in six DR and DC β-chain sequences, as well as in seven murine class II sequences (three I-Aβ and four I-Aα alleles) were analyzed. The results suggest that the mutation rates are of a comparable magnitude in the nucleotide sequences encoding the first and second external domains of the class II molecules. Nevertheless, the allelic amino acid replacements are predominantly located in the first domains. We conclude that a conservative selective pressure acts on the second domains, whereas in many positions in the first domains replacement substitutions are selectively neutral or maybe even favoured. Thus, the difference between the first and second domains as regards the number of amino acid replacements is mainly due to selection.

Key words: major histocompatibility complex/class II antigens/amino acid sequence/polymorphism

Introduction

A fundamental trait of higher organisms is their ability to discriminate between self and nonself. Even primitive fungi are capable of identifying and destroying nonself. Self-nonself recognition systems in invertebrates and vertebrates display similar characteristics: recognition molecules on the cell surface, effector mechanisms aiming at the destruction of nonself and a considerable polymorphism in the recognition structures (Hildemann *et al.*, 1981). In mammals, a self-nonself recognition system is encoded by a chromosomal segment called the major histocompatibility complex (MHC). This genetic region has a central role in the immune system (Benacerraf, 1981). Two types of extensively polymorphic cell surface recognition structures, denoted histocompatibilty antigens, are encoded by loci within the MHC. Class I antigens, present on virtually all nucleated cells, act as restricting elements when cytotoxic T cells detect foreign antigens (nonself) in the context of self (Zinkernagel and Doherty, 1979). Class II antigens are restricting elements which permit helper T cells to recognize foreign antigens in the context of self on the surface of macrophages, B cells and certain T cells (Shevach and Rosenthal, 1973; Katz and Benacerraf, 1976).

The murine class II antigen region (the I region) seems to contain only two loci encoding conventional class II antigens, the I-A locus and the I-E locus (Hood *et al.*, 1983). The number of loci encoding human class II antigens is not known. Serological data have firmly established the existence of three loci: DR, DC and SB, and possibly a fourth locus called BR (Tosi *et al.*, 1978; Shaw *et al.*, 1980; Tanigaki and Tosi, 1982). Genomic hybridization data and the number of isolated cDNA and genomic clones encoding class II antigen subunits are consistent with about twice as many loci (Böhme *et al.*, 1983: Larhammar *et al.*, 1983b; Auffray *et al.* 1983b; Trowsdale *et al.*, 1983). Since it is likely that all class II antigens serve as restricting elements, although with varying efficiency (Thorsby *et al.*, 1982), the redundancy of class II antigens and their genetic polymorphism provide the population with a large pool of genes encoding similar molecules with identical functions. This genetic polymorphism is probably of functional significance.

The class II antigens are composed of two membrane-integrated subunits, called α and β. The structures of human as well as murine α- and β-chains have been elucidated (Kratzin *et al.*, 1981; Larhammar *et al.*, 1982a, 1983a, 1983b; Auffray *et al.*, 1982; Korman *et al.*, 1982; Lee *et al.* 1982; Yang *et al.*, 1982; Long *et al.*, 1983; Choi *et al.*, 1983; Malissen *et al.*, 1983; Saito *et al.*, 1983; Benoist *et al.*, 1983a, 1983b; Hyldig-Nielsen *et al.*, 1983; Mathis *et al.*, 1983; McNicholas *et al.*, 1983). The α and β chains display the same major features. The extracellular part consists of two domains, each encompassing ~90 amino acid residues. The second domains of the α- and β-chains are homologous to each other, as well as to β_2-microglobulin, the third domain of class I antigen heavy chains and immunoglobulin constant domains. The β-chains of all class II antigens studied are polymorphic (Charron and McDevitt, 1979; Cook *et al.*, 1979) as are the human DCα (Auffray *et al.*, 1983a) and the murine I-Aα chains (Benoist *et al.*, 1983a, 1983b).

The present investigation was undertaken to obtain more information on the nature of the class II antigen polymorphism. Possible causes of the polymorphism are discussed in the light of this information.

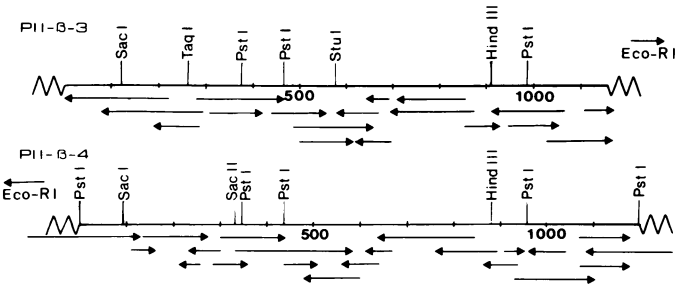

Fig. 1. Restriction maps of the pII-β-3, and pII-β-4 cDNA inserts. The arrows denote the sequencing strategy. Orientations in plasmid vectors are indicated by assignment of the insert end closest to the *Eco*RI site of pBR322.

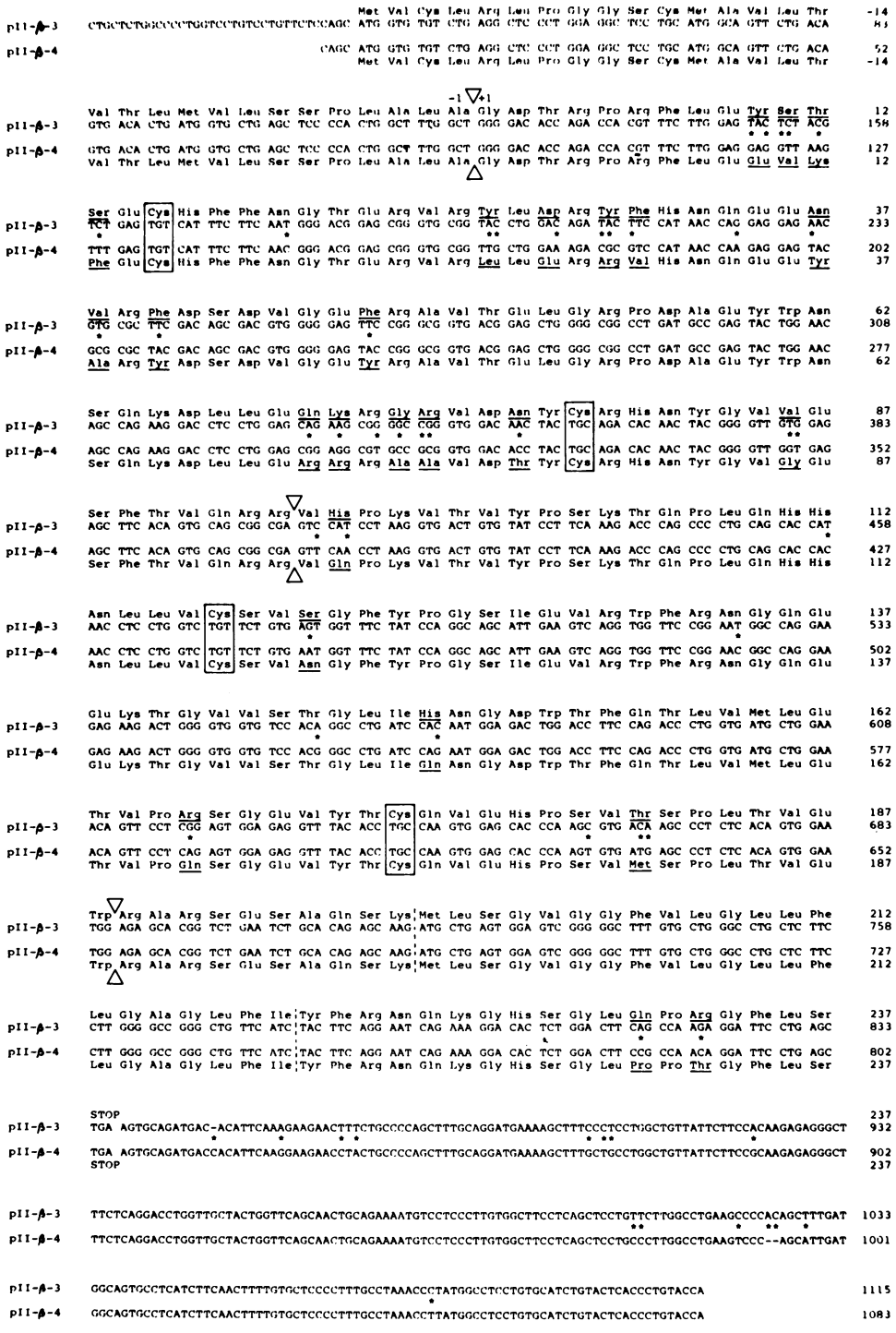

Fig. 2. Nucleotide and predicted amino acid sequences of pII-β-3 and pII-β-4. Stars denote nucleotide differences between the two sequences. Amino acid replacements are underlined. The number 1 in the amino acid sequence refers to the beginning of the mature protein. Cysteine residues involved in disulfide bonds are enclosed within boxes. The membrane-spanning hydrophobic segment is indicated by dashes. The open arrows indicate the boundaries of the first and second external domains.

Results and Discussion

The human species is well outbred and therefore suitable for studies of polymorphism. Four human class II antigen β-chain nucleotide sequences have previously been determined: those of the two DCβ cDNA clones pII-β-1 (Larhammar *et al.*, 1982b) [this clone, previously referred to as pDR-β-1, has been renamed to avoid confusion with the DRβ clones] and pII-β-2 (Schenning *et al.* 1984) and of the DCβ gene cosII-102 (Larhammar *et al.* 1983b) as well as that of the DRβ cDNA clone HLA-DRβ1 (Long *et al.*, 1983). In order to obtain more data we isolated and sequenced two additional DRβ cDNA clones, pII-β-3 and pII-β-4 (Figures 1 and 2). These clones were obtained from the same DR 3/w6 Raji cell line library as pII-β-1 and pII-β-2. HLA-DRβ1 was derived from a cell typed to be DR4/w6 and cosII-102 from a DR,Dw4/4 homozygous library. In addition to these sequences the amino acid sequence of a DR2 β-chain has been published (Kratzin *et al.*, 1981).

The murine I-A and I-E loci each contain a single α and a single β gene (Steinmetz *et al.*, 1982; Hood *et al.*, 1983). The situation is more complex in the human class II antigen region, since it seems to contain considerably more genes (Peterson *et al.*, 1983). Thus, the DC locus contains two β genes. Structural analyses of these genes have shown that although the exons encoding their second domains and membrane segments are highly homologous, other portions of the genes are distinctly different (Larhammar *et al.*, 1983b; Hyldig-Nielsen *et al.*, in preparation). From these analyses it can be concluded that pII-β-1, pII-β-2 and cosII-102 form an allelic series.

The DR locus also contains more than one β gene as evidenced by analyses of cDNA (Long *et al.*, 1983) and genomic clones (Larhammar *et al.*, in preparation). However, the coding nucleotide sequences of the three DRβ clones pII-β-3, pII-β-4 and HLA-DRβ1 are at least as similar to each other as are the three allelic DCβ clones (Table I). Moreover, the amino acid sequences predicted from the three DRβ

Table I. Percent nucleotide differences between DC and DR β-chain nucleotide sequences

	DCβ		DRβ		
	pII-β-2	cosII-102	pII-β-3	pII-β-4	HLA-DRβ1
DCβ: pII-β-1	8.0	4.2	28.9	28.2	28.8
pII-β-2		7.3	29.6	28.8	29.1
cosII-102			29.5	28.4	28.9
DRβ: pII-β-3				5.6	4.2
pII-β-4					5.3

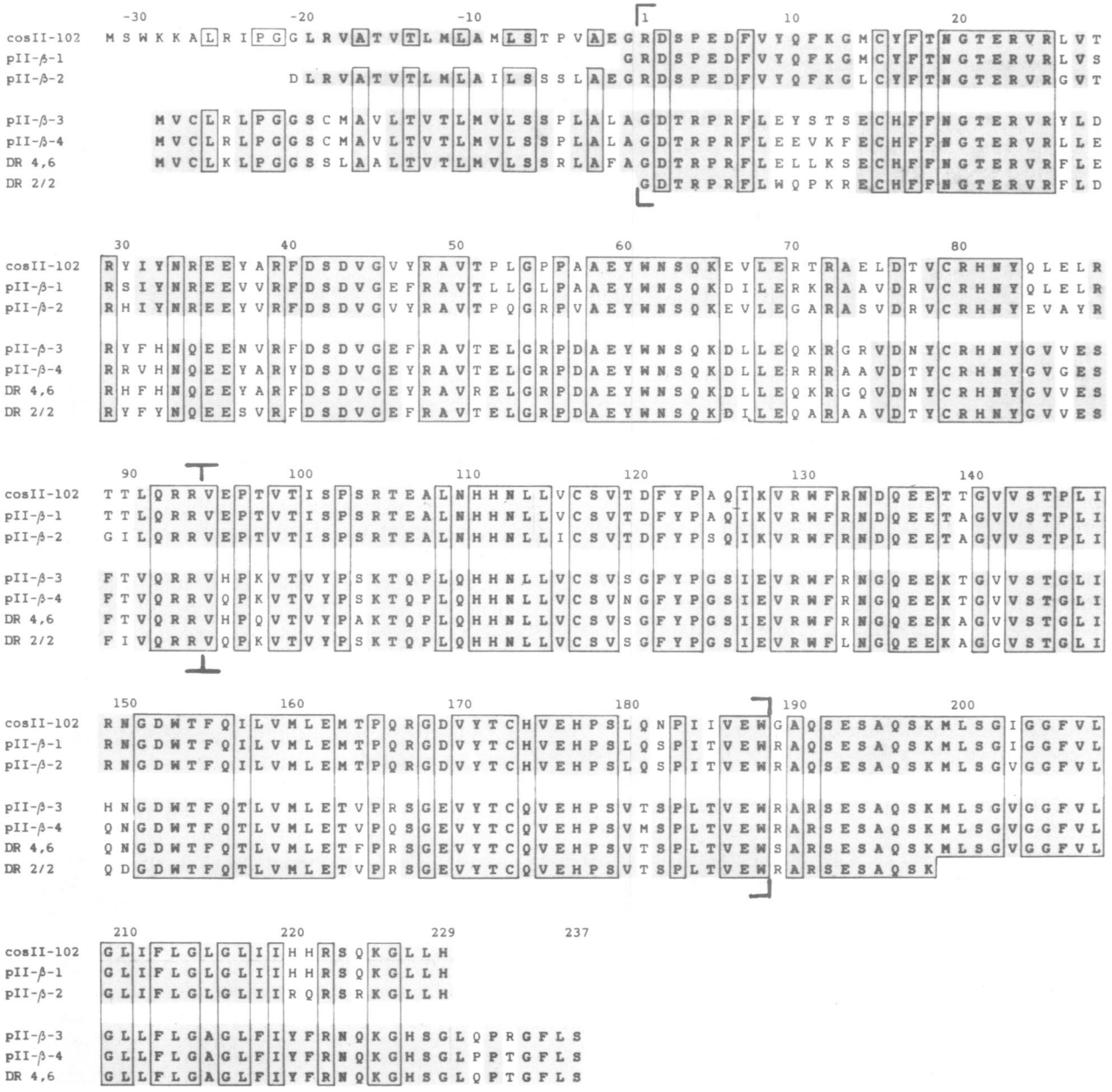

Fig. 3. Comparison of the β-chain amino acid sequences predicted from cosII-102, pII-β-1,-2,-3,-4, HLA-DRβ1 (DR 4,6) and the protein sequence of a DR 2/2 β chain. Boxes denote amino acid identity between all seven chains. Shadowed residues are identical within the DC or the DR group of sequences. Numbers indicate positions of amino acids in the mature protein. The boundaries of the first and second external domains are indicated by square brackets.

clones also are as homologous to each other as are the sequences deduced from the DCβ alleles (Figure 3). These data are consistent with the three DRβ cDNA clones belonging to the same allelic series. However, the possibility remains that the clones are derived from DRβ genes that are pseudoallelic, i.e., as homologous to each other as true alleles. Such a situation exists in the murine class I antigen D,L region where molecular analysis of the genes have shown that the serologically defined segregation of D and L specificities may be difficult to entertain (Maloy and Coligan, 1982; Evans *et al.*, 1982). The distinction between alleles and pseudoalleles is of even less significance if closely related genes participate in intergenic conversion events, as has been suggested for the class I antigens (Pease *et al.*, 1983; Weiss *et al.*, 1983a, 1983b). We conclude that pII-β-3, pII-β-4 and HLA-DRβ1 either form an allelic series or are derived from pseudoallelic genes. In either case the sequences can be used in comparisons to find out the distribution of polymorphism within the DRβ molecules.

Comparison of amino acid sequences

A comparison of the primary structures of four DRβ and three DCβ polypeptide chains allows the identification of positions in the sequences where amino acid replacements are prevalent. When DCβ chains are compared with DRβ chains, the amino acid replacements are almost equally distributed along the entire length of the molecules. In contrast, within each group of sequences, the second domain, the membrane-spanning portion, and the cytoplasmic tail are conserved (Figure 3). The conservation of these portions of the β-chains may be due to specific interaction with their respective types of α-chains. Although homologous, DRα and DCα chains differ considerably from each other (Larhammar *et al.* 1982a; Auffray *et al.*, 1982). The differences between the conserved parts of the DRβ and DCβ chains may also suggest that the two types of class II antigens fulfill somewhat different physiological functions.

Within each β-chain group most of the amino acid replacements are located in the amino-terminal domain (Figure 3). Of the 23 amino acid differences between the extracellular portions of the DRβ chains encoded by pII-β-3 and pII-β-4, 18 are located in the first domain and five in the second domain. The number of replacements found between the DCβ alleles pII-β-1 and pII-β-2 are 22 and two for the first and second domains, respectively. Similar distributions of amino acid replacements were found in the other possible DRβ to DRβ and DCβ to DCβ comparisons (Figure 3).

It has been shown that allelic amino acid replacements in the murine Aβ (Choi *et al.*, 1983) and Aα (Benoist *et al.*, 1983b) class II genes occur preferentially in the first of the two external domains. The present data extend this observation to the human DRβ and DCβ genes, suggesting that it is a general property of polymorphic class II molecules. The class II antigens are known to be structurally related to immunoglobulins (Larhammar *et al.*, 1982a, 1982b) and their organization into a variable first and a constant second domain is reminiscent of the variable and constant immunoglobulin domains.

The homology to immunoglobulins raises the question of whether there is a mechanism which generates variability specifically within the first domain. This is the case for immunoglobulins where V-J joining (Brack *et al.*, 1978), somatic mutations (Bothwell *et al.*, 1981; Crews *et al.*, 1981) and possibly also gene conversion (Baltimore, 1981) contribute to the polymorphism of the variable domain. There is no evidence of somatic rearrangements involving class II genes, but a mechanism such as sequence-specific mutagenesis could conceivably contribute specifically to first domain variability. In that case, the difference between the first and second domains would be analogous to the difference between the polymorphic Aα and non-polymorphic Eα genes, for which it has been suggested that variations in the mutation rate may play a role (Benoist *et al.*, 1983b). Similarly, the frequency of gene conversion involving short pieces of DNA (Pease *et al.*, 1983; Weiss *et al.*, 1983a, 1983b) could be higher in the first domain. Alternatively, the conservation of the second domain could be the result of gene conversion involving the entire exon. Indeed, it has been suggested that certain parts of the human class I genes may be homogenized by such a mechanism (Biro *et al.*, 1982). Finally, it is possible that the difference between the first and second domains is due to selection at the phenotypic level (see below). An answer to these questions can be obtained by comparing the silent substitution rates in the two domains.

A second set of questions deals with the selective pressures acting on the polymorphic class II molecules. The homology of class II antigens to immunoglobulins suggests that the polymorphism of the first domain may be selectively advantageous. Such selection would tend to increase the frequency of amino acid replacements. If, on the other hand, any one of the two domains is subject to a conservative selective pressure, replacement substitutions should be underrepresented in that domain. These questions can be answered by comparing the silent and replacement substitution rates within each domain.

Comparison of nucleotide sequences

We wished to determine whether the observed polymorphism of class II antigens is compatible with a simple model of evolution based on mutations and selection. To assess mutation rates and selective pressures, the six DRβ and DCβ nucleotide sequences were analyzed with respect to the distribution of silent and replacement substitutions. The sequences of three murine Aβ alleles (Choi *et al.*, 1983; Larhammar *et al.*, 1983a) and four complete Aα alleles (Benoist *et al.*, 1983b) were also included in the analysis. To collect the largest possible set of independent data, we aligned the three DRβ sequences to each other and counted the total number of substitutions of each type within the two extracellular domains. Wherever one sequence differed from the other two, one substitution was scored; where all three sequences differed, two substitutions were scored. The same procedure was repeated independently for the DCβ, Aβ and Aα sequences. The data are given in Table II.

The data compiled in this way do not represent the actual number of substitutions that has occurred within any single evolutionary lineage. Instead, they are a sample of the total number of substitutions that have occurred in several allelic or pseudoallelic sequences that have descended from a common ancestral gene. This kind of data can provide information on the average distribution of substitutions with respect to type and position within each group of related sequences.

Mutation rates

The number of silent substitutions in a translated DNA sequence is believed to reflect the local mutation rate, since they usually occur with frequencies comparable with those of substitutions in small introns and adjacent flanking sequences

Table II. Distribution of observed substitutions and potential substitution sites in 13 human and murine class II transplantation antigen sequences

Domain	First domain				Second domain			
Locus	DRβ	DCβ	Aβ	Aα	DRβ	DCβ	Aβ	Aα
Observed number of silent substitutions	7	12	4	5	9	7	11	9
Averge number of silent sites	67.6	64.6	63.8	55.3	64.9	65.6	68.2	62.7
Silent substitutions in % of silent sites	10.4	18.6	6.3	9.0	13.9	10.7	16.1	14.4
Observed number of replacements	28	34	27	25	9	6	4	3
Average number of replacement sites	214.4	217.4	203.2	208.8	217.1	216.4	213.8	219.4
Replacements in % of replacement sites	13.1	15.6	13.3	12.0	4.1	2.8	1.9	1.4

In the calculations, first domains are defined as amino acid residues 1–94 of the mature DRβ and DCβ polypeptides and as residues 6–94 and 1–88 of the Aβ and Aα polypeptides. The second domains are residues 95–188 of the β-chains and 89–182 of the α-chains.

Table III. Replacement substitutions in class II transplantation antigens: comparison of observed numbers to numbers expected to occur in the absence of selection

Domain	First domain				Second domain			
Locus	DRβ	DCβ	Aβ	Aα	DRβ	DCβ	Aβ	Aα
Total number of substitutions (n)	35	46	31	30	18	13	15	12
Expected number of replacements in a random sample of n substitutions (r)	26.6 ± 2.5	35.4 ± 2.9	23.6 ± 2.4	23.7 ± 2.2	13.9 ± 1.8	10.0 ± 1.5	11.4 ± 1.7	9.4 ± 1.4
Observed number of replacements	28	34	27	25	9	6	4	3
Significant difference?	No	No	No	No	Yes	Yes	Yes	Yes
Level of significance	–	–	–	–	0.013	0.017	0.0001	0.0002

The expected number of replacements is given as the mean and standard deviation of the corresponding binomial distribution (see Materials and methods).

(Perler *et al.*, 1980; Efstratiadis *et al.*, 1980; Miayata *et al.*, 1980). Thus, a comparison of the number of silent substitutions in the two external domains will reveal whether their mutation rates are the same. To obtain a sequence-independent estimate of the silent substitution frequency in each domain, the number of silent substitutions (Table II) was expressed in percent of the average number of potential silent sites (Perler *et al.*, 1980).

The ratio of the silent substitution frequency in the first domain to that in the second domain then provides a measure of domain-specific variation in the mutation rate. If no such variation exists the numbers obtained should be close to unity. For the DRβ, DCβ, Aβ and Aα sequences the ratios are 0.75, 1.74, 0.39 and 0.63. Because of the small number of silent substitutions within each group, none of these numbers reflects a statistically significant deviation from unity. More significantly, the weighted average of the four ratios (with respect to the number of silent substitutions) is close to unity, 0.93. This figure, which is based on the distribution of 64 silent substitutions within 13 sequences, strongly suggests that there is no systematic domain-specific variation in the mutation rates.

Selective pressures

To assess the effects of selection we computed the replacement substitution frequencies within the four groups of sequences (Table II). Unlike the silent substitutions, the replacement substitutions are very unevenly distributed, being much more frequent in the first domain. This is most probably due to selection, since the mutation rate appears to be the same in the two domains (see above). The excess of replacements in the first domain could be due to a conservative selective pressure acting on the second domain and/or a positive selection for polymorphism in the first domain. To test these possibilities, we compared the observed number of replacements in each domain with the number expected to occur in the absence of selection (Table III). The statistical significances of the differences between the observed and expected numbers were computed (see Materials and methods for details).

The data in Table III show that the observed numbers of replacements in the second domains are significantly lower than expected for all four groups of class II chains. Therefore, it can be concluded that a conservative selective

pressure acts on the second domains, eliminating replacement substitutions. In contrast, the number of replacements in the first domains do not deviate significantly from the numbers expected to occur in the absence of selection. Again, the data are consistent for all four groups of sequences. Thus, there is no evidence of a positive selection for polymorphism. Still, it is conceivable that some structurally important residues are selectively conserved also in the first domains, whereas at other positions polymorphism is selected for, resulting in an average replacement frequency characteristic of an unselected segment. This would be in line with the suggested clustering of polymorphic residues in the first domain of the Aα molecule (Benoist *et al.*, 1983b).

Conclusions

The aim of the present investigation was to elucidate the role of mutation rates and selective pressures in the generation of class II histocompatibility antigen polymorphism. To this end we compared allelic or pseudoallelic sequences at the human DRβ and DCβ and the murine Aβ and Aα loci. In total, 200 nucleotide substitutions in 13 cDNA and genomic sequences were included in the analysis. Our interpretation of the data supports the following general conclusions. (i) There is no evidence of an intrinsically higher mutation rate in the variable first domain of the class II molecules. The two external domains accumulate silent subtitutions at comparable rates. (ii) The constant second domains are subject to conservative selective pressures at the polypeptide level, eliminating most replacement substitutions. (iii) The variable first domains behave like unselected DNA sequences. Thus, silent and replacement substitutions accumulate in proportion to the total number of potential silent and replacement sites.

The importance of selective pressures at the protein level for the development of the differences between the first and second domains of the class II antigens seem to be evident. Thus, it is conceivable that the genetic polymorphism of these molecules is generated by multiple independent point mutations, many of which are saved if they occur in the first domain and eliminated if they occur in the second domain. This does not necessarily mean that all point mutations in a given sequence have originated within the same gene. It is possible that a mutation may occur in one gene and then be copied to another by gene conversion. It has indeed been argued that such events may contribute to the genetic polymorphism of class I histocompatibility antigens (Pease *et al.*, 1983; Weiss *et al.*, 1983a, 1983b).

The class II antigens differ from the polymorphic class I antigens in that sequences from different class II loci form well-defined groups with respect to homology (e.g., see Table I). Therefore, it is unlikely that genetic information is frequently exchanged between different class II loci. Still, gene conversion could occur between the alleles or pseudoalleles within each class II locus. We conclude that if such events contribute to the polymorphism of class II antigens, then the mechanism does not discriminate between the two external domain exons, since their silent substitution frequencies are of a comparable magnitude. This applies also to mechanisms such as sequence-specific mutagenesis or sequence homogenization by gene conversion involving entire exons. Thus, the pronounced difference as regards polymorphism between the first and second external domains of the class II molecules appears to be due to selection at the phenotypic level.

Materials and methods

Isolation of pII-β-3 and pII-β-4

A nick-translated 627-bp *Aval* fragment of pII-β-1 was used as a probe in colony hybridization (Gergen *et al.*, 1979) to a cDNA library consisting of 20 000 clones.

Characterization of plasmids

Plasmid DNA was isolated by CsCl/ethidium bromide equilibrium gradient centrifugation followed by sucrose gradient centrifugation. Restriction maps were constructed by single and double digestions. Nucleotide sequence determination was carried out according to Maxam and Gilbert (1980) on fragments labeled at a single 5′ (Maxam and Gilbert, 1980) or 3′ (Tu and Cohen, 1980) end.

Calculations of silent and replacement substitutions

The number of potential silent and replacement sites were calculated separately for each nucleotide sequence as described by Perler *et al.* (1980). The values were then averaged for each group of sequences. An observed substitution was assessed as replacement only if it could not be regarded as silent. The calculated substitution frequencies were not corrected for multiple substitutions since the total number of substitutions was low and since the data were not used to estimate evolutionary distances.

Statistical analysis

A statistical procedure was used to test whether the observed number of replacement substitutions in any given domain nucleotide sequence differs significantly from the number of replacement substitutions expected to occur in the absence of selection. To obtain an estimate of the latter number, the following two assumptions were made: (i) each nucleotide substitution is derived from a single point mutation event, (ii) in the absence of selection, point mutations are distributed at random among the total number of potential silent and replacement sites. Under these circumstances the expected number of replacement substitutions, r, will have a binomial distribution $\binom{n}{r}(R/T)^r(1-R/T)^{n-r}$, where n is the total number of observed substitutions, R is the number of potential replacement sites (Table II) and T is the total number of sites (silent and replacement). The significance levels in Table III were computed from such distributions; they are the probabilities that r will assume values less than or equal to the observed numbers of replacement substitutions.

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