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Organization and evolution of *C4* and *CYP21* genes in primates: importance of genomic segments

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Abstract. The evolutionary relationship between two central major histocompatibility complex (MHC) genes, *C4* and *CYP21*, was investigated by employing pulsed field gel electrophoresis (PFGE) and conventional restriction fragment length polymorphism (RFLP) analyses in human and nonhuman primates. Using *Taq* I in conjunction with *C4* and *CYP21* probes, it has been found that there are four major types of *C4* genes [defined by 7.0, 6.4, 6.0, and 5.4 kilobases (kb) *Taq* I fragments] and two major types of *CYP21* genes (3.7 and 3.2 kb fragments) in human and nonhuman primates including chimpanzee, gorilla, and orangutan. All of the eight possible combinations of *C4* and *CYP21* genes can be identified on one or more human ancestral haplotypes (*AH*). It is concluded that each of the major types of *C4* and *CYP21* (and each of the combinations between these) predated human speciation. PFGE analysis with *Mlu* I and *Pvu* I suggested that each *C4* + *CYP21* segment has a specific length of 30–50 kb and that each *AH* carries one, two, three, or even more segments. In the case of *C4*, it is important to note that there is no simple relationship between the RFLP and the protein classifications. Thus, at least some of the expressed polymorphisms could be relatively recent in that they are carried by the same or different gene types. These findings are consistent with the hypothesis that MHC *AH*s have been formed from a large pool of specific genomic segments and that further haplospecific polymorphism has developed subsequently.

Introduction

Complement *C4* genes are closely linked to the steroid 21 hydroxylase (*CYP21*) genes and located approximately halfway between the class I and class II gene clusters within the major histocompatibility complex (MHC). In humans the numbers of the *C4* and *CYP21* genes vary depending upon MHC ancestral haplotypes (*AH*) but there are always equal numbers of *C4* and *CYP21* genes (Carroll et al. 1986; Schneider et al. 1986; Collier et al. 1989; Zhang et al. 1990; Tokunaga et al. 1991).

Usually human *C4* genes are classified as if there were a two-locus (*C4A* and *C4B*) system (Schneider et al. 1986). However, some alleles cannot be classified (Yu and Campbell 1987; Schneider et al. 1990; Tokunaga et al. 1991). In the chimpanzee a two-locus model was proposed based on C4 allotyping (Granados et al. 1987) and there is no doubt that there are two major types of protein corresponding, in part, to *C4A* and *C4B* (Christiansen et al. 1991). However, restriction fragment length polymorphism (RFLP) analyses in the chimpanzee and other nonhuman primates (Dawkins et al. 1989, 1991a; Kawaguchi et al. 1990) yielded some unexpected results. For example, chimpanzees often (or always) possess a 6.4 kilobases (kb) *Taq* I fragment which we (Garlepp et al. 1986) and others (Carroll et al. 1986; Schneider et al. 1986) had considered to be a specific marker for the *8.1 AH* possibly reflecting a deletion of most of *C4A*, all of *CYP21A* and some of *C4B*.

We have used techniques including C4 allotyping and DNA typing by conventional gel electrophoresis and pulsed field gel electrophoresis (PFGE) to clarify: 1) How many categories of *C4* and *CYP21* genes can be defined in various primates. 2) Whether there are differences in gene copy number of *C4* and *CYP21* in nonhuman primates. 3) Whether there are *C4* and *CYP21* genes carried together as a genomic segment. 4) Whether there is evidence of recent deletion and/or insertion.

Materials and methods

Cell lines. Thirteen Epstein Barr virus (EBV)-transformed human homozygous cell lines, including ten studied during the 10th Interntional

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Table 1. Human cell lines homozygous for the *AH* used in this study.

AH	WSID*	*HLA A*	*Cw*	*B*	*C2*	*Bf*	*C4A*	*C4B*	*DR*	*DQ*	Race†
7.1	9082	3	7	7	C	S	3	1	15	6	C
7.2		24	7	7	C	S	3+3	1	1	5	M
8.1	9022	1	7	8	C	S	*Q0*	1	3	2	C
18.2	9085	30	5	18	C	F1	3	*Q0*	3	2	C
35.2	9006	3	4	35	C	F	3+2	*Q0*	1	5	C
42.1	9021	-	2	42	C	F	1	*Q0*	3	4	N
44.1	9090	2	5	44	C	S	3+3	*Q0*	4	7	C
44.3	9050	29	-	44	NT	S	*Q0*	1	7	2	C
47.1	9047	3	6	47	C	F	A91	*Q0*	7	2	C
52.1		24	-	52	C	S	3+2	*Q0*	15	6	M
57.1	9052	2	6	57	C	S	6	1	7	9	C
65.1		-	8	65	C	S	2	1+2	1	5	C
60.X	9069	2	10	60	NT	NT	NT	NT	8	4	C

* WSID = 10th International MHC Testing Workshop identity number.
† Race: C = Caucasoid; M = Mongoloid; N = Negroid.
- = not defined, NT = not tested, X = not defined *AH*.

Histocompatibility Workshop, were used in this study. Local cell lines were genotyped for *HLA* class I, II, and complement loci (Table 1). Cell lines of seven chimpanzees (*Pan troglodytes*), one gorilla (*Gorilla gorilla*), and one orangutan (*Pongo pygmaeus*) were kindly provided by R. Bontrop (ITRI-TNO Primate Centre, Rijswijk, The Netherlands) and by P. Parham (Stanford University, California, USA). Genomic DNA was prepared from the cell lines according to the standard phenol/chloroform/isoamyl alcohol method.

Human cDNA probes. The *C4* (pAT-A) and *CYP21* (pC21/3c) cDNA probes have been described previously (Belt et al. 1984; White et al. 1984a; Zhang et al. 1990). The probes were labeled with α-[^{32}P]-dCTP by random priming (Feinberg and Vogelstein 1983).

Complement and HLA allotyping. Allotyping of complement components Bf, C2, and C4 was performed as described previously (Alper et al. 1972, 1976; Sim and Cross 1986; Zhang et al. 1988). *HLA A, B, C, DR,* and *DQ* alleles were determined by standard microcytotoxicity techniques using defined antisera obtained from local and international workshops.

Conventional Southern blot analysis. Briefly, genomic DNA was digested to completion with *Taq* I (Promega, Cleveland, OH) according to the manufacturer's instruction. The digests were electrophoresed on 0.8% agarose gels at 20 V for ~40 h in 1 × tris borate (TBE) buffer at room temperature. Detailed methods are provided elsewhere (Southern 1975; Tokunaga et al. 1991; Wu et al. 1992).

Pulsed field gel electrophoresis (PFGE) analysis. Preparation of genomic DNA in agarose blocks, digestion with *Mlu* I and *Pvu* I, separation of digests by PFGE and Southern hybridization were performed as previously described (Anand et al. 1986; Tokunaga et al. 1988; Zhang et al. 1990).

Evaluation of gene copy number. The quantitation of the gene copy number by densitometry is detailed by Zhang and co-workers (1990).

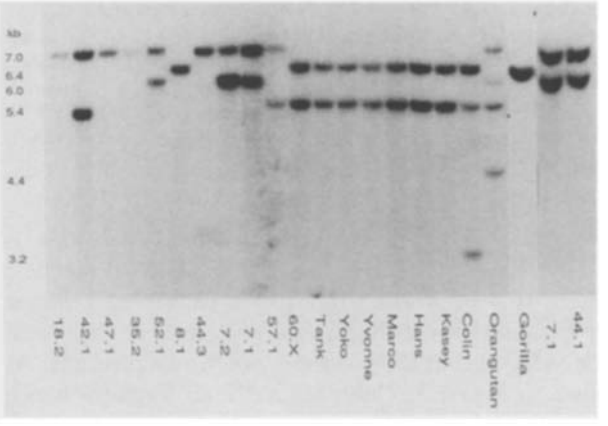

Fig. 1. *C4/Taq* I RFLP analysis of genomic DNA from human and nonhuman primates shows the presence of different types of *C4* genes. Four distinct fragments, 7.0, 6.4, 6.0, and 5.4 kb, are present representing four major types of the *C4* genes. In the *7.2 AH* an O. D. ratio of 0.5 (7.0:6.0 kb) suggests a duplication of the 6.0 kb *Taq* I *C4* gene. The *35.2 AH* displays a single 7.0 kb fragment but C4 allotyping demonstrated two distinct allotypes being C4A3 and A2 (Table 1) indicating that both *C4* genes are marked by the 7.0 kb *Taq* I fragment (Fig. 5). The *44.1 AH* exhibits 7.0 and 6.0 kb fragments indicating the presence of two *C4* alleles (C4A3+3) on this *AH* (Table 1; Tokunaga et al. 1991). All chimpanzees except Colin and one human (*60.X*) carry 6.4 kb and 5.4 kb fragments with similar intensities. These contrast with the *8.1 AH* which has the 6.4 kb fragment alone. Additional fragments were found in Colin (3.2 kb fragment also hybridizing to CYP21, see Figure 2) and the orangutan (4.4 kb). In Colin O. D. ratios of 1.9:1.2:1.0 (6.4:5.4:3.2 kb) indicate a duplication of the 6.4 kb *C4* gene. The orangutan shows four fragments with O. D. ratios of 1.9:1.0:2.2:2.2 (7.0:6.0:5.4:4.4 kb) suggesting a single copy of the 6.0 kb but two copies of 7.0, 5.4, and 4.4 kb fragments. The gorilla carries a single 6.4 kb fragment identical to that of human *8.1 AH*.

Results

There are four major types of C4 genes existing in human and other primates. *C4/Taq* I RFLP analysis has revealed four distinct fragments, 7.0, 6.4, 6.0, and 5.4 kb in both human and nonhuman primates (Fig. 1). With only one exception (Colin) all chimpanzees have a composite pat-

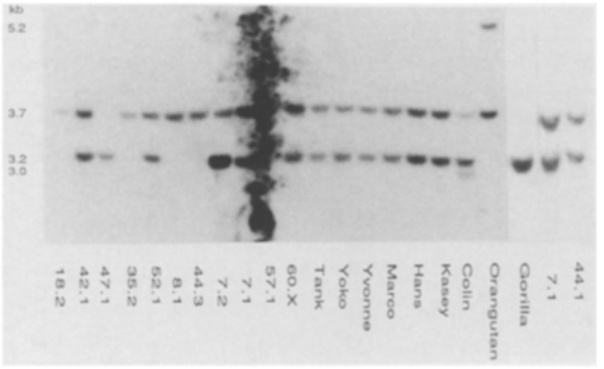

Fig. 2. *CYP21/Taq* I RFLP reveals the presence of two major types of *CYP21* genes in human and nonhuman primates. The same blots as in Figure 1 were stripped and reprobed for *CYP21*. Two additional types of *CYP21* genes (3.0 kb in Colin and 5.2 kb in the orangutan) are present. The human *60.X* is identical to all chimpanzees except Colin with 3.7 kb (*CYP21-3.7*) and 3.2 kb (*CYP21-3.2*) of similar intensities. An O. D. ratio of 0.5 (3.7:3.2 kb) in the *7.2 AH* again indicates a duplication of *CYP21-3.2*. In Colin O. D. ratios of 1.0:2.1:0.8 (3.7:3.2:3.0 kb) suggest at least an extra copy carried by the 3.2 kb fragment as compared with 3.7 and 3.0 kb fragments. In the orangutan, a ratio of 0.7 (5.2:3.7 kb) suggests 2:3 or 3:4 copies (Zhang et al. 1990) of *CYP21* genes. Human *47.1 AH* associated with 21 hydroxylase deficiency (Fleischnick et al. 1983; White et al. 1984a) carries only the *CYP21-3.2* gene. Interestingly, the gorilla appears to be homozygous for *CYP21-3.2* but must have a functional *CYP21* gene. The *57.1 AH* was tested previously (Dawkins et al. 1989).

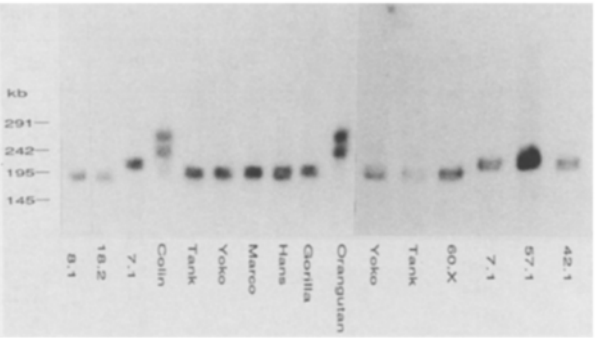

Fig. 3. Length differences by PFGE suggest differences in gene copy number as well as gene sizes. Genomic DNA was digested with *Mlu* I, subjected to PFGE separation, and probed for *C4*. It can be seen that human *8.1* and *18.2 AH*s which carry single *C4* and *CYP21* genes have fragments of ~180 kb while *7.1*, *57.1*, and *42.1 AH*s which carry two copies for each yield fragmenets of ~210 kb indicating a difference of some 30 kb. The chimpanzees Tank, Yoko, Marco, Hans, the gorilla, and the human *60.X* carrying two short *C4* genes as defined by the 6.4 and 5.4 kb *Taq* I fragments have PFGE fragments of approximately 195 kb. Apparently Colin and the orangutan are heterozygous as indicated by the 240 kb and 270 kb fragments which may carry more than two copies of *C4* + *CYP21* segments. Note that human *7.1*, Yoko, and Tank were repeated as standards.

tern of 6.4 + 5.4 kb fragments with similar intensities. A human individual (*60.X*, Table 1) displays an identical pattern (6.4 + 5.4 kb) suggesting a conserved genomic organization between human and chimpanzee. Additional types of *C4* genes are present in nonhuman primates. As shown in Figure 1, Colin has a 3.2 kb fragment which also carries the *CYP21* gene (see Fig. 2). The orangutan carries a *C4* gene marked by the 4.4 kb fragment and densitometric ratios of C4 fragments within the orangutan have revealed two copies of *C4* genes marked by the 7.0, 5.4, and 4.4 kb fragments (Fig. 1).

Two major types of CYP21 genes are present in all primates. Hybridization of the same *Taq* I blots with the *CYP21* probe (Fig. 2) has demonstrated that two major types of *CYP21* genes marked by 3.2 kb (*CYP21-3.2*) and 3.7 kb (*CYP21-3.7*) are present in all primates included in this study. In addition, other types of *CYP21* genes have also been identified in Colin (3.0 kb) and the orangutan (5.2 kb), these have not been observed in humans to date. The gorilla has a single 3.2 kb *Taq* I fragment indicating homozygosity for *CYP21-3.2*.

PFGE analysis allows recognition of gene copy number, gene size, and genomic segments of C4 + CYP21. *C4/Taq* I RFLP (Fig. 1) has revealed that the *8.1* and *18.2 AH*s carry single copies of *C4* and *CYP21* genes while the *7.1*, *57.1*, and *42.1* AHs carry two of each. As shown in Figure 3 and Table 2, PFGE/*Mlu* I analysis suggests a difference of ~30 kb between the haplotypes with one copy of the *C4* and *CYP21* genes compared with those having two copies of these genes. PFGE analysis with a different enzyme (*Pvu* I, see Figure 4) reveals a difference of ~50 kb between the haplotypes carrying one or two copies of the *C4* and *CYP21* genes confirming the observation with *Mlu* I. Using *Mlu* I, PFGE gives a specific genomic length (195 kb) to most chimpanzees and a human haplotype (*60.X*) which carry two short forms of *C4* genes, both 16 kb long as defined by the 6.4 kb and 5.4 kb *Taq* I fragments (Yu et al. 1986; Palsdottir et al. 1987; Kawaguchi et al. 1990).

The single 6.4 kb *C4* fragment and 3.2 kb *CYP21* fragment seen in the gorilla may suggest single *C4* and *CYP21* genes. However, PFGE with *Mlu* I and *Pvu* I analyses showed that this primate has genomic fragments of the same length as in chimpanzees, carrying two copies of *C4* + *CYP21* and larger than human *AH*s carrying a single copy of *C4* + *CYP21*, e. g., *AH*s *8.1* and *18.2* (Figs. 3 and 4). Therefore, it is possible that the gorilla has two copies of *C4* + *CYP21* segment (6.4 kb *C4* + 3.2 kb *CYP21*; Fig. 5). The results from *Taq* I RFLP and PFGE using two informative enzymes, *Mlu* I and *Pvu* I (Dunham et al. 1989; Tokunaga et al. 1991), suggest that the orangutan may carry 3–4 copies of *C4* + *CYP21* genomic segments on its haplotypes (Fig. 5). As shown in Figure 3, Colin carries two large PFGE/*Mlu* I fragments similar to

Table 2. Comparison of *C4* and *CYP21* RFLPs between human and nonhuman primates by conventional and PFGE analyses (size in kb).

Name	Species	*Taq* I/pAT-A						*Taq* I/pC21/3c				*Mlu* I pAT-A	*Pvu* I pC21/3c
		7.0	6.4	6.0	5.4	4.4	3.2	5.2	3.7	3.2	3.0		
18.2	human	+							+			180	350
47.1	human	+								+		-	-
44.3	human	+							+			180	-
35.2	human	+							+			-	-
7.1	human	+		+					+	+		210	390
44.1	human	+		+					+	+		-	390
52.1	human	+		+					+	+		-	390
7.2	human	+		++					+	++		-	440
57.1	human	+			+				+	+		210	390
42.1	human	+			+				+	+		210	-
65.1	human	+			++				+	++		-	400
8.1	human		+						+			180	340
60.X	human		+		+				+	+		195	-
Tank	chimpanzee		+		+				+	+		195	390
Yoko	chimpanzee		+		+				+	+		195	390
Yvonne	chimpanzee		+		+				+	+		-	-
Marco	chimpanzee		+		+				+	+		195	390
Hans	chimpanzee		+		+				+	+		195	390
Kasey	chimpanzee		+		+				+	+		-	-
Colin*	chimpanzee		++		+		+		+	++	+	270	
												240	-
Machi	gorilla		+							+		195	390
CP81*	orangutan	++		+	++	++		++	+++			270	-
												240	480

* Colin and the orangutan are heterozygous as indicated by *Taq* I and PFGE (*Mlu* I).
+ = fragment present, + + = fragment present with doubled intensity
- = not tested

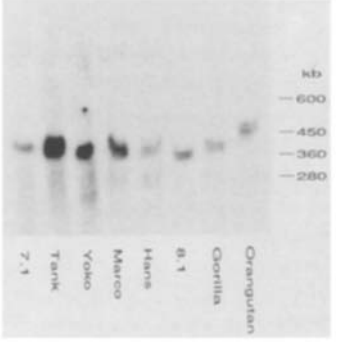

Fig. 4. PFGE analysis by *Pvu* I confirms the differences in length observed by *Mlu* I/PFGE analysis. The same samples as in Figure 3 were digested with *Pvu* I and probed for *CYP21*. As shown the chimpanzees (Tank, Yoko, Marco, and Hans) and the gorilla have fragments of ~390 kb similar to human *7.1 AH* which carries two copies of C4 + CYP21. The human *8.1 AH* which carries a single copy of *C4 + CYP21* shows a smaller fragment of ~340 kb as compared with the chimpanzees, gorilla, and *7.1 AH*. Again it can be seen that the orangutan carries a much larger fragment (~480 kb) than the others (though Colin was not tested with *Pvu* I) suggesting three or more copies of *C4* and *CYP21* genes on its haplotypes (see also Figures 1 and 3).

the orangutan suggesting more than two copies of *C4 + CYP21* segments but *C4/Taq* I RFLP is less informative in this respect (Fig. 1).

A model taking account of the *Taq* I RFLP and PFGE data is presented in Figure 5. This suggests that the correlation between the *C4* gene RFLP and protein classifications as well as *C4* and *CYP21* gene arrangements may be explained by the presence of one or more primordial *C4 + CYP21* segments of some 30–50 kb each which are found in human and nonhuman primates.

Discussion

By using the *Taq* I and probing for *C4* and *CYP21*, we have shown that there are four major types of *C4* genes defined by 7.0, 6.4, 6.0, and 5.4 kb *Taq* I fragments and two major types of *CYP21* genes defined by 3.2 and 3.7 kb fragments present in human and nonhuman primates studied. Others (Bontrop et al. 1991) have reported similar results to those shown here. Additional types of both *C4*

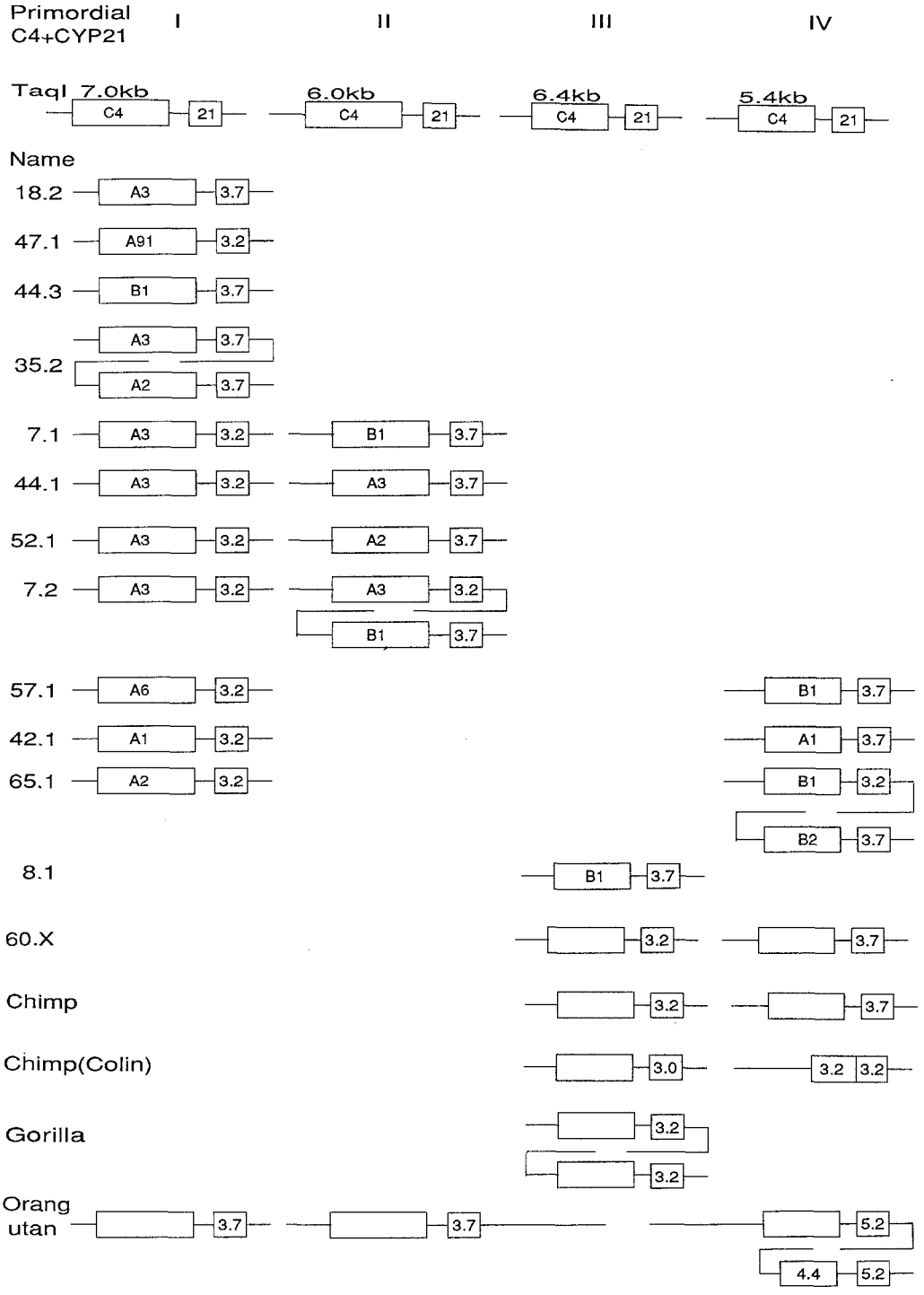

Fig. 5. Schematic presentation of *C4* and *CYP21* gene arrangements which may be explained by several primordial genomic segments. Four major types of *C4* genes can be recognized by *Taq* I and their major accompanying *CYP21* genes (*CYP21-3.2* and *CYP21-3.7*) vary depending upon *AHs*. Thus, a *C4* gene and a *CYP21* gene combines to form a primordial *C4+CYP21* genomic segment of 30–50 kb in length as defined by PFGE. Four major types of *C4* genes are used to identify such segments and there are two different segments under each category. The presence of such genomic segments is depicted for representative haplotypes. The *broken lines* between the segments are introduced since the genomic lengths between them are not known. Most chimpanzees are homozygous for the haplotype as shown (chimp). However, Colin is heterozygous with one haplotype the same as that found in most chimpanzees and the second haplotype is as shown (Colin). On this second haplotype both *C4* and *CYP21* genes are marked by the 3.2 kb fragment suggesting that they could be fused genes. An alternative possibility is that they are separate genes with an identical marker. The orangutan is heterozygous with one possible haplotype carrying four *C4+CYP21* segments as shown but the other haplotype may carry only three segments and lack a segment carrying 6.0 kb *C4* and *CYP21B* genes (see Figures 1 and 2). The *65.1 AH* was tested previously (Garlepp et al. 1986; Tokunaga et al. 1991).

and *CYP21* genes have also been observed in nonhuman primates (Table 2). A human individual (*60.X*) carries the same C4 6.4 + 5.4 kb and CYP21 3.7 + 3.2 kb *Taq* I patterns present in most chimpanzees (Figs. 1 and 2) suggesting a similar *C4* + *CYP21* organization (Fig. 5).

PFGE is useful in evaluating differences in gene copy number and in gene size (Dunham et al. 1989; Zhang et al. 1990). We have used PFGE and two informative enzymes to determine the number of genomic segments present on different *AH*s and have shown that differences in length by PFGE relate to differences in the number of genomic segments carrying *C4* + *CYP21* genes (Tokunaga et al. 1991). As shown here, human *AH*s (e. g., *8.1* and 18.2) carrying single copies of *C4* and *CYP21* genes have PFGE fragments shorter by some 30–50 kb than those (e. g., *7.1*, *57.1*, and *42.1*) carrying two copies of each (Figs. 3 and 4, Table 2). Therefore, the difference of ~30 kb by *Mlu* I or ~50 kb by *Pvu* I between these haplotypes actually reflects a genomic segment carrying one copy of *C4* + *CYP21* (Fig. 5).

This is also the case in nonhuman primates. As shown in Figures 3 and 4 and summarized in Table 2, all the chimpanzees except Colin carry fragments of ~195 kb by *Mlu* I and ~390 kb by *Pvu* I. Combining both *Taq* I RFLPs and PFGE analyses, the results indicate two *C4* + *CYP21* segments in the chimpanzees. With two different enzymes, PFGE revealed that the gorilla may carry two probably identical *C4* + *CYP21* segments (Fig. 5). The orangutan has two large PFGE fragments and informative *Taq* I RFLP (see Fig. 1) suggesting the presence of 3–4 *C4* + *CYP21* segments on its haplotypes (Fig. 5). Colin may carry more than two *C4* + *CYP21* segments as suggested by PFGE (Fig. 3) but *C4/Taq* I RFLP is less informative in this respect (Fig. 1).

In species other than primates, *C4* and *CYP21* genes may also be carried as segments with variation in copy number depending upon haplotype. In the mouse, for example, *H-2* haplotypes can carry two, three, or even four copies of *C4* (or *Slp*) + *CYP21* segments (White et al. 1984b; Levi-Strauss et al. 1985; Tosi et al. 1985). This may also be true in sheep and cattle (Skow et al. 1988; Ren et al. 1991), but further confirmation is required.

It is important to note that *C4* genes classified as different by the *Taq* I approach can encode the same allotype (e. g., C4B1). Contrariwise, one type of *C4* gene (e. g., 7.0 kb) can express completely different allotypes, e. g., C4A3 or C4B1 (Fig. 5). It appears that at least some of the expressed C4 polymorphisms may be relatively recent in that they are carried by the same or different gene types. Sequencing the *C4d* region in primates (Kawaguchi et al. 1992) and comparison within and between species are consistent with this conclusion. Thus, it is not surprising that there is no simple relationship between the *C4* type by RFLP and by protein allotyping (Tokunaga et al. 1991).

Table 3. Eight possible combinations of different *C4* and *CYP21* genes are identified on one or more human *AH*s studied. Three of these combinations are shared between human and nonhuman primates (see also Figure 5).

		C4/Taq I			
	kb	7.0	6.4	6.0	5.4
		7.1	*60.X*	*7.2*	*65.1*
		7.2	gorilla		
		42.1	chimpanzee		
	3.2	*44.1*			
		47.1			
		52.1			
		57.1			
CYP21/Taq I		*65.1*			
		18.2	*8.1*	*7.1*	*42.1*
		35.2		*7.2*	*57.1*
	3.7	*44.3*		*44.1*	*65.1*
				52.1	*60.X*
				orangutan	chimpanzee

To date, the evidence available provides no direct support for recent deletion and/or insertion of individual *C4* and *CYP21* genes as suggested previously (Garlepp et al. 1986; Carroll et al. 1986; Schneider et al. 1986). Rather, it appears that a *C4* gene and a *CYP21* gene [and perhaps other genes, see Morel and co-workers (1989)] are carried on a primordial genomic segment which has been retained as an intrinsic component of a particular *AH*. For those haplotypes carrying two or more linked segments, each segment carries different types of *C4* and/or *CYP21* genes as determined by *Taq* I RFLP. The exceptions (which could represent segment duplication) would appear to be unusual (e. g., *35.2*) at least during primate evolution.

As shown in Table 3, all of the eight possible combinations of *C4* and *CYP21* genes can be identified on one or more human *AH*s. Three such combinations are shared between human and nonhuman primates. Each combination was probably present in an ancient pool as proposed in Figure 5. It is concluded that each of these major types of *C4* and *CYP21* genes (and each of combinations between these) predated human speciation and some could have existed prior to primate speciation. These findings are consistent with the hypothesis that MHC *AH*s have been formed from a large pool of specific genomic segments and further haplospecific protein polymorphism has developed subsequently (Dawkins et al. 1991b).

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