NULL ALLELES OF HUMAN COMPLEMENT C4

Evidence for Pseudogenes at the *C4A* Locus and for Gene Conversion at the *C4B* Locus

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C4 is the only component of the human complement system that is coded for by two genes, *C4A* and *C4B*, showing 99% homology. The genes for the two C4 isotypes are located with the genes for the second component (C2), factor B (BF), and steroid 21-hydroxylase (21-OHA and 21-OHB) between HLA-B and -DR in the MHC on chromosome six (1–4). The *C4* and *21-OH* genes are tandemly arranged and have probably arisen by duplication (Fig. 1 *a*). Based on the direction of transcription, C4A is usually expressed at C4 locus I, whereas C4B is usually expressed at locus II (5).

C4A and C4B are highly polymorphic with more than 35 alleles including null alleles (*C4Q0*) at both loci (6). The polymorphism can be defined by electrophoretic mobility of the intact protein or its subunits (7–9), by serology of Rodgers (Rg) and Chido (Ch) determinants (10), and by functional studies of complement activation and binding characteristics (11, 12). A sequence of four amino acids in the C4d region (Chido 4 determinant on C4B molecules) is responsible for the major structural and functional differences of the C4 isotypes (Fig. 1 *c*; 9, 11–13). The antigenic determinants Rodgers and Chido are generally expressed on the C4A and C4B isotypes, respectively, but rare reversed associations have been described (14). Gene conversion has been discussed as a possible mechanism for the generation of aberrant allotypes (15, 16).

Null alleles of *C4A* or *C4B* (*AQ0* or *BQ0*) occur at frequencies of 0.1–0.3 in the normal population (17), and are assessed by the absence of gene products. The structural analysis of the *C4* genes at the DNA level has revealed that only a proportion of *C4* null alleles result from gene deletions affecting an entire *C4* gene and one adjacent *21-OH* gene, and consequently other null alleles were due to nonexpressed genes (pseudogenes) (18, 19). Alternatively, it has been suggested that null alleles represent the expression of two identical, and therefore undistinguishable, allotypes on a haplotype, and this results from gene conversion of *C4A* to *C4B* and the reverse (5, 20).

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Haplotypes with the typical C4 two-gene configuration expressing two different C4A allotypes (e.g., *C4A3,2 BQ0*), as well as a single *C4A3 BQ0* haplotype with two C4 loci only containing specific sequences of the C4A isotype, have already been reported (5, 20–22).

To further characterize the structural basis of C4 null alleles not accounted for by deletions, we studied 26 individuals homozygous either for *C4AQ0* or for *C4BQ0*. Haplotypes carrying null alleles not due to deletions were selected for study by genomic RFLP analysis. Using synthetic oligonucleotides, restriction fragment analysis, and direct sequencing of genomic DNA amplified by the polymerase chain reaction (23, 24), we studied the isotype-specific sequences of the C4d region that correlate with the Rodgers and Chido determinants as defined by serology. The presence of a respective DNA sequence for a given determinant not detectable by serology would indicate the nonexpression of an allele, whereas the correlation of DNA sequence and serological findings would provide evidence for the possible expression of both adjacent C4 genes. Using this approach, we detected six haplotypes in which *C4A* genes were nonexpressed and 19 haplotypes with apparent nondeleted *BQ0* alleles which had only C4A-specific sequences. These findings provide evidence for independent genetic mechanisms at the C4 loci resulting either in pseudogenes or in genes which, as a result of gene conversion, may express the reverse isotype.

Materials and Methods

Selection of Individuals and Genetic Typing. 26 individuals with homozygous C4A or C4B deficiency (*AQ0* or *BQ0*) were included. All individuals had been haplotyped for MHC class I, II, and III gene products in family studies according to the following methods: HLA-A, -B, -C, and -DR antigens were assigned by the microlymphocytotoxicity assay (25). C4 typing was carried out by high voltage agarose gel electrophoresis in a discontinuous buffer system after desialation of the samples with neuraminidase, followed by immunofixation or hemolytic overlay (7). C4 null alleles were confirmed by C4 α chain typing using SDS-PAGE (9). The serological typing of the C4 antigenic determinants Rodgers and Chido was carried out on all C4BQ0 individuals by means of hemagglutination inhibition with human alloantisera as described (10).

DNA Probes. The C4-specific probe used for Southern blot analysis was a 400-bp Bam HI/Kpn I fragment from the 5′ end of the full-length C4 cDNA clone pAT-A (26), and the 21-OH-specific probe was a 900-bp Bgl I/Kpn I fragment from the genomic cosmid clone cos 1E3 (2).

Southern Blot Analysis. Human genomic DNA was prepared from 10-ml blood samples (27). DNA samples of 10 μg were digested with Taq I at a concentration of 5 U/μg (Boehringer Mannheim, Mannheim, FRG). The DNA fragments were electrophoresed in a 0.8% agarose gel and blotted onto nitrocellulose membrane (BA 85; Schleicher & Schuell, Dassel, FRG). The C4 and 21-OH probes were radiolabeled with α-[^{32}P]dCTP (Amersham Buchler, Braunschweig, FRG) by nick translation. Hybridization of blots and interpretation of restriction fragment patterns were carried out as described previously (19).

PCR Amplification. The polymerase chain reaction was performed as described (24) using 1 μg genomic DNA and 2 U Taq polymerase (Cetus Corp., Emeryville, CA). The temperature cycles were modified as follows and repeated 35 times: 2 min at 91°C (denaturing), 3 min at 55°C (annealing), 5 min at 70°C (extension).

The primer sequences were chosen according to published C4 gene sequences (28). The four oligonucleotide primers were as follows (cf., Figs. 1 *b* and 2 *a*): L-1, 5′-TGAGTTTGGCCCAGGAGCAG-3′; L-2, 5′-TGCCACAGTCTCATCATTGC-3′; L-3, 5′-TGCGGATCCAGCAGTTTCGGAAG-3′; L-4, 5′-ATAGGATCCTAAGGTCCCCTGGGCCT-3′. With these primers, the C4d regions of all C4 (A and B) genes present in an individual are amplified.

Detection of C4 Isotype-specific Sequences. For oligonucleotide hybridization (Fig. 1), the

amplified DNA was electrophoresed in a 1% agarose gel. After transfer to nylon membrane (Hybond N; Amersham Buchler), the blots were hybridized first with oligo A according to procedures described previously (29). After autoradiography for 6 h, the probe was removed by boiling the blots for 20 min in 0.1% SDS and rehybridized with oligo B. Oligos A and B were endlabeled with polynucleotide kinase (Boehringer Mannheim) with γ-[^{32}P]ATP (Amersham Buchler).

For the direct analysis of restriction fragments (Fig. 2), the amplified DNA (using PCR primers (L3/L4) was purified from PCR buffer components by adsorption to glass milk (Geneclean; BIO 101; La Jolla, CA), and digested with 5 U EcoO 109, with 3 U Nla IV, or with 10 U Alu I (all from New England Biolabs, Bad Schwalbach, FRG). DNA digested with Nla IV and EcoO 109 was electrophoresed in a 1.5% agarose gel. DNA digested with Alu I was separated in a 10% polyacrylamide gel, as the resulting fragments were smaller than 100 bp. The fragments were visualized on a UV light box at 312 nm after ethidiumbromide staining.

DNA Sequencing. For direct sequencing of PCR-amplified genomic DNA, the amplified DNA fragment was purified by agarose gel electrophoresis followed by electroelution to remove the PCR primers. The eluted template DNA was further purified by adsorption to glass milk (see above). The sequencing reaction was carried out following a protocol for double-stranded DNA with the Sequenase™ sequencing system (United States Biochemical Corp., Cleveland, OH) and α-[^{35}S]dATP (Amersham Buchler). As sequencing primers, the following oligonucleotides were used: L-3; L-4 (see above); L-5, 5'-TGTTGAAGGTCCTGAGT-3'; and L-6, 5'TTTGGTGGGCAATGATG-3' (28).

Results and Discussion

To identify carriers of nondeleted *AQ0* and *BQ0* alleles, we determined the *C4* gene structure by Southern blot analysis of Taq I–digested DNA (not shown). Four Taq I fragments can be identified with the 5' C4 probe. A 7-kb fragment usually represents a *C4A* gene at locus I, whereas the 6- and 5.4-kb fragments identify long and short genes at locus II; these are usually *C4B*. A 6.4-kb fragment represents a short *C4B* gene in combination with a deletion of the *C4A* gene, whereas *C4B* gene deletions are detected on the assessment of different intensities of the C4A- and C4B-specific DNA fragments (19). Two smaller Taq I fragments are detected with the 21-OH–specific probe. A 3.2-kb fragment represents a *21-OHA* gene and a 3.7-kb fragment a *21-OHB* gene (30). All haplotypes with C4 gene deletions are listed in Table I. In 5 of 11 *AQ0* individuals both *C4A* genes were deleted, whereas in 6 only one *C4A* gene was deleted and the other was present. 2 of 15 *BQ0* individuals had both *C4B* genes deleted, 7 had one gene deleted, and in 6 neither was deleted. 10 of 16 *C4A* gene deletions were linked to the extended MHC haplotype *HLA-A1*, *Cw7*, *B8*, *DR3*, *C4AQ0B1*, whereas no characteristic haplotypes were found to be linked with *C4B* gene deletions (Table I). 19 individuals with 25 haplotypes carrying nondeleted null alleles, 6 *AQ0* and 19 *BQ0*, were selected for the study.

The antigenic determinant Ch4 is present on all C4B molecules and therefore defines the C4B isotype. Oligonucleotides (oligo A, oligo B; Fig. 1 *c*), capable of distinguishing the isotype-specific Ch4 sequence at the DNA level, were used to screen nondeleted *C4* null alleles. As hybridization of these oligonucleotides to genomic Southern blots did not give satisfactory results, we used PCR with subsequent oligonucleotide hybridization (Fig. 1). A 258-bp sequence of the C4d region containing the five isotype-specific base substitutions was amplified with primers L1 and L2 (Fig. 1, *b–c*). Under the given hybridization conditions, the C4A and C4B isotype-specific 23-mers hybridized exclusively with the respective C4d sequence (cf., con-

TABLE I

Haplotypes with C4 Gene Deletions

HLA-				C4		Taq I fragments				
A	C	B	DR	A	B	C4A	21-OHA	C4B	21-OHB	No.
							kb			
A. C4A/21-OHA Deletions:										
1	w7	8	3	Q0	1	–*	–	6.4	3.7	(10)
1		8	2	Q0	1	–	–	6.4	3.7	(1)
1		8	4	Q0	1	–	–	6.4	3.7	(1)
1		8	5	Q0	3	–	–	6.4	3.7	(1)
1	w2	35	3	Q0	1	–	–	6.4	3.7	(1)
1		w70	3	Q0	1	–	–	6.4	3.7	(1)
3		w70	4	Q0	1	–	–	6.4	3.7	(1)
B. 21-OHA/C4B Deletions:										
24		8	1	3	Q0	7.0	–	–	3.7	(1)
24	w5	18	3	3	Q0	7.0	–	–	3.7	(1)
2		18	4	3	Q0	7.0	–	–	3.7	(1)
24	w1	27	3	3	Q0	7.0	–	–	3.7	(1)
1 (11)	w4	35	1	3	Q0	7.0	–	–	3.7	(3)
2		w65	1	3	Q0	7.0	–	–	3.7	(1)
C. C4B/21-OHB Deletions:										
3	w6	w47	7	1	Q0	7.0	3.2	–	–	(1)
2		w60	w6							
				3	Q0	7.0	3.2	–	–	(1)

* –, A deletion of the respective gene.

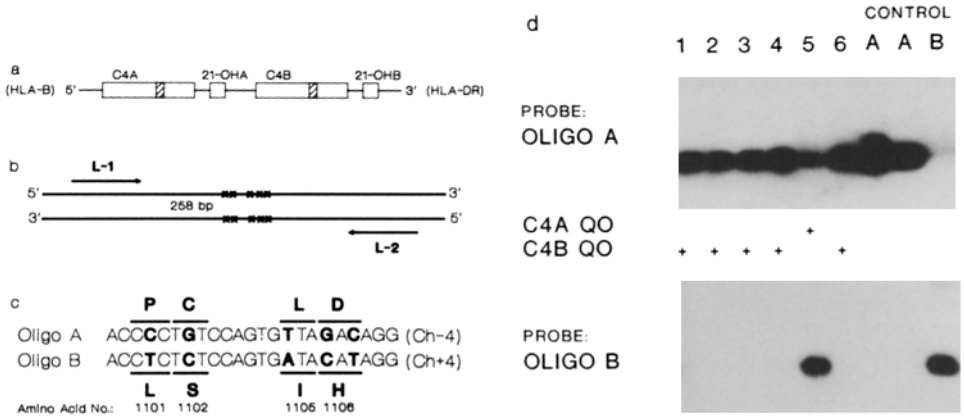

FIGURE 1. (*a*) Map of the tandemly arranged *C4* and *21-hydroxylase* genes of the MHC class III region. The orientation relative to *HLA-B* and *-DR* is shown. The hatched areas within the *C4* genes depict the location of the polymorphic C4d regions. (*b*) Strategy for PCR amplification of the C4d isotype-specific sequence. The positions of polymorphic nucleotides are indicated by x (26). (*c*) Oligonucleotide sequences A and B used to detect the isotype-specific sequences of the *C4* genes. The isotypic nucleotide positions and amino acid residues are indicated by bold characters and bars. (*d*) Examples for hybridization of oligos A and B with amplified 258-bp fragment of homozygous *C4AQ0* (lane *5*) and *C4BQ0* individuals (lanes *1–4*, *6*) with at least one nondeleted *C4Q0* allele. Controls A and B represent amplified DNA from genomic cosmid clones cos 3A3 (*C4A* gene) and KEM-1 (*C4B* gene) (gift of Dr. M. C. Carroll, Harvard Medical School, Boston, MA).

trols A and B, Fig. 1 *d*). All six haplotypes carrying *AQ0* alleles with expressed *C4B* genes reacted with oligos A and B. In contrast, all 19 haplotypes with *BQ0* alleles and expressed *C4A* genes reacted with oligo A alone (Fig. 1 *d*; Table II).

Taq I fragments of 6.0 and 5.4 kb generally represent *C4B* genes at C4 locus II (5, 19), but the nonhybridization of oligo B with genes at locus II suggested that C4A sequences were present. This result could also be explained by point mutations or deletions in the Ch4 sequence not detectable by Southern blot analysis. Therefore, we used RFLP analysis of amplified DNA with Nla IV, which also distinguishes between *C4A* and *C4B* genes (reference 5; Fig. 2 *a*), and confirmed that all *BQ0* individuals revealed only C4A-specific fragments (Fig. 2 *b*, Table II). In addition, the Rgl- and Chl-specific DNA sequences, which can be distinguished by RFLP analysis with EcoO 109 (5; Fig. 2 *a*), also corroborated the data with oligos A and B. All six haplotypes with *AQ0* alleles revealed both the Rgl- and Chl-specific fragments. All 19 haplotypes with *BQ0* alleles had only the Rgl-specific fragment (Fig. 2 *b*, Table II). The plasma samples of all *BQ0* individuals were tested for the presence of Rg/Ch determinants by serology, and in accordance with the DNA data, Chl was absent from all *BQ0* individuals (data not shown).

The determinant Ch6, which is normally expressed by C4B but also known on C4A (14), was assessed on the protein and the DNA level as well. Amplified DNA

TABLE II

HLA Haplotypes, C4 Gene Structure and Rodgers/Chido Determinants of Nondeleted C4Q0 *Alleles*

HLA alleles		C4 alleles		Taq I fragment		Oligo A B Nla IV		Eco0 109		Alu I		
- B	- DR	A	B	A	B	Ch - 4	Ch + 4	Rg + 1	Ch + 1	Ch - 6	Ch + 6	No.
				kb								
A. C4AQ0 B1 (2) haplotypes:												
w60	w6	Q0	1	7.0	5.4	+	+	+	+		nt	(2)
w60	w6	Q0	2	7.0	5.4	+	+	+	+		nt	(3)
w60	3	Q0	2	7.0	5.4	+	+	+	+		nt	(1)
B. C4A3 BQ0 haplotypes:												
44	4	3	Q0	7.0	6.0	+	-	+	-	+	-	(4)
44	5 (w6)	3	Q0	7.0	6.0	+	-	+	-	+	-	(2)
35	1	3	Q0	7.0	6.0	+	-	+	-		*	(3)
35	w6	3	Q0	7.0	6.0	+	-	+	-		*	(1)
18	3	3	Q0	7.0	6.0	+	-	+	-		*	(1)
39	4	3	Q0	7.0	6.0	+	-	+	-	-	+	(1)
w60	1	3	Q0	7.0	6.0	+	-	+	-	+	-	(1)
w70	w6	3	Q0	7.0	6.0	+	-	+	-		*	(1)
C. C4A3,2 BQ0 haplotypes:												
35	1	3,2	Q0	7.0	6.0	+	-	+	-	-	+	(1)
35	1	3,2	Q0	7.0	6.0	+	-	+	-		*	(1)
35	4	3,2	Q0	7.0	6.0	+	-	+	-	-	+	(1)
w62	w6	3,2	Q0	7.0	6.0	+	-	+	-	-	+	(1)
w62	1	3,2	Q0	7.0	6.0	+	-	+	-		*	(1)

nt, not tested, as nonexpression has already been shown.

* Individual DNA heterozygous for Ch6, assignment to haplotype or gene not possible.

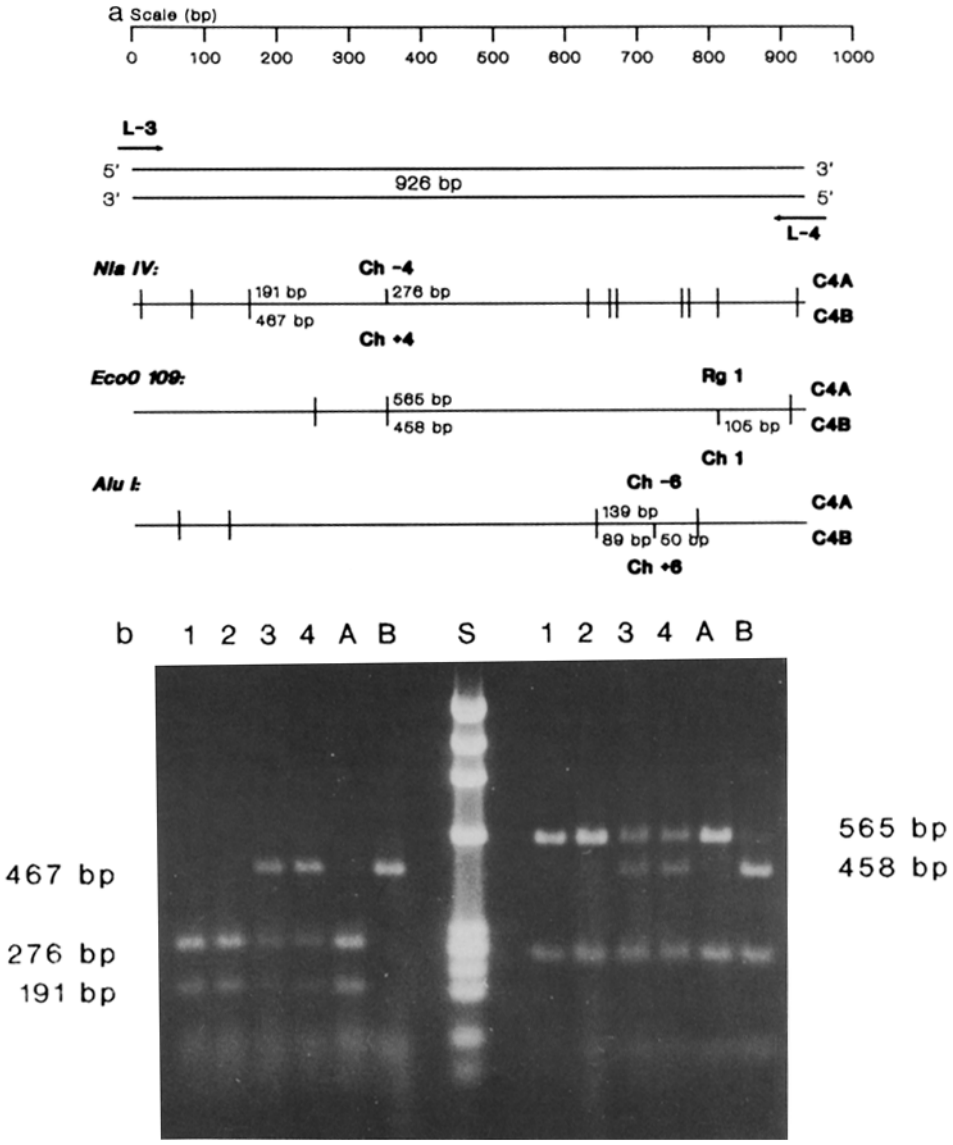

FIGURE 2. Legend on facing page.

from *BQ0* individuals was digested with Alu I (Fig. 2, *a*, *c*). The results were not as uniform as for the Ch4 and Ch1 determinants. A number of heterozygotes were detected at the DNA level (indicated by asterisk in Table II; cf., Fig. 2 *c*, lanes *1*, *2*), which did not allow the assignment of the Ch−6 and Ch+6 sequences to one of the haplotypes. Seven MHC haplotypes identified by linkage to *HLA-B44* and *Bw60* lacked the sequence coding for Ch6 at both C4 loci (Table II; Fig. 2 *c*, lanes *3*, *5*). However, four haplotypes linked to *HLA-B35*, *B39*, and *Bw62* had the sequence for Ch6 at both loci (Table II; Fig. 2 *c*, lanes *4*, *6*). Again, these findings were confirmed by serological studies: when the Ch−6 sequence was detected at

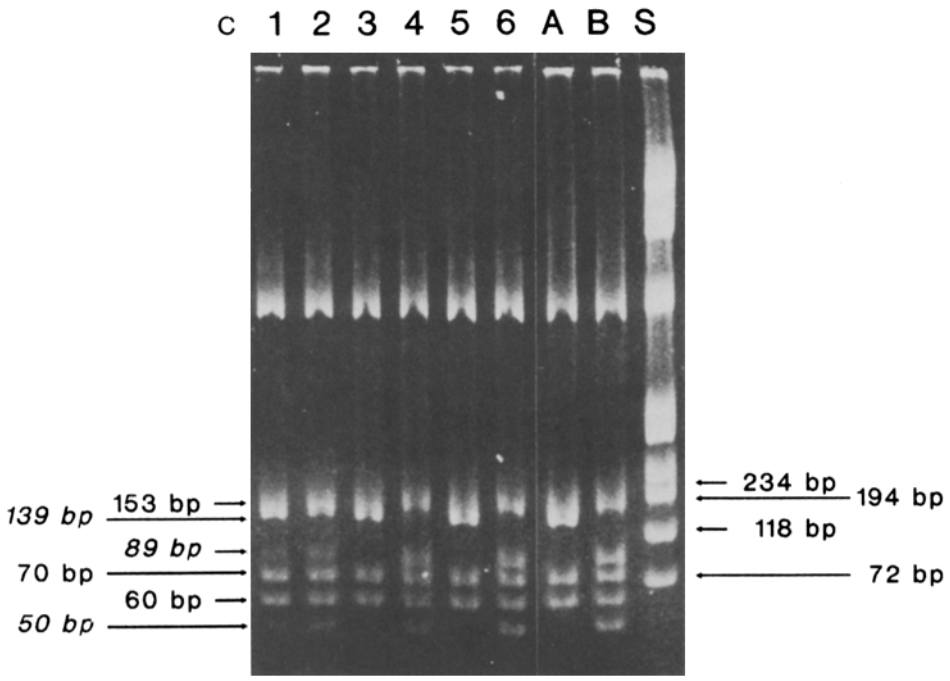

FIGURE 2. (*a*) Strategy for the amplification and RFLP analysis of the 926-bp C4d fragment to analyze the sequences coding for determinants Ch4 (Nla IV), Rgl/Ch1 (EcoO 109), and Ch6 (Alu I). The fragment sizes for each enzyme indicating the presence of C4A- or C4B-specific sequences are given in the respective restriction maps (5, 26). (*b*) Examples for restriction fragment analysis of PCR-amplified DNA with Nla IV (Ch4; *left*) and EcoO 109 (Rgl/Ch1; *right*). Fragment sizes are indicated on both sides. The DNA fragment length standard (S) is Hae III-digested Phi X 174 bacteriophage DNA. (Lanes *1*, *2*) DNA from *C4BQ0* individuals; (lanes *3*, *4*) DNA from *C4AQ0* individuals; (lanes *A*, *B*) C4A, C4B controls (cf., Fig. 1 *d*). (*c*) Examples for restriction fragment analysis of PCR-amplified DNA from *C4BQ0* individuals with Alu I (Ch6). Fragment sizes of genomic fragments are indicated on the left side with the polymorphic fragments printed in italics, and sizes of the DNA fragment length standard (as in Fig. 2 *b*) are given on right side. (Lanes *1*, *2*) DNA from individuals with Ch-6 and Ch+6 sequences (marked by asterisk in Table II), note the difference in fragment intensities between 60/70 bp and 50/89 bp; (lanes *3*, *5*) DNA from individuals with Ch-6 sequence; (lanes *4*, *6*) DNA from individuals with Ch+6 sequence; (lanes *A*, *B*) C4A, C4B controls (cf., Fig. 1 *d*).

the DNA level, the determinants Rg1 and 2 were present on the C4 molecules. When the Ch6 sequence was detected, only Rg1 and Ch6 but not Rg2 were found by serology (data not shown). This reversed antigenic association of C4A alleles with the Ch6 determinant has already been described for several C4A allotypes (14) and thus does not contradict our previous results.

For a single individual homozygous for *HLA-B44*, *C4A3 BQ0*, and with four structurally intact C4 genes we directly sequenced the relevant portions of the C4d region after PCR amplification. We only detected sequences typical for the C4A isotype, i.e., Ch-4 (Fig. 3 *a*), Rg1 and Ch-6 (Fig. 3 *b*), thus confirming our results

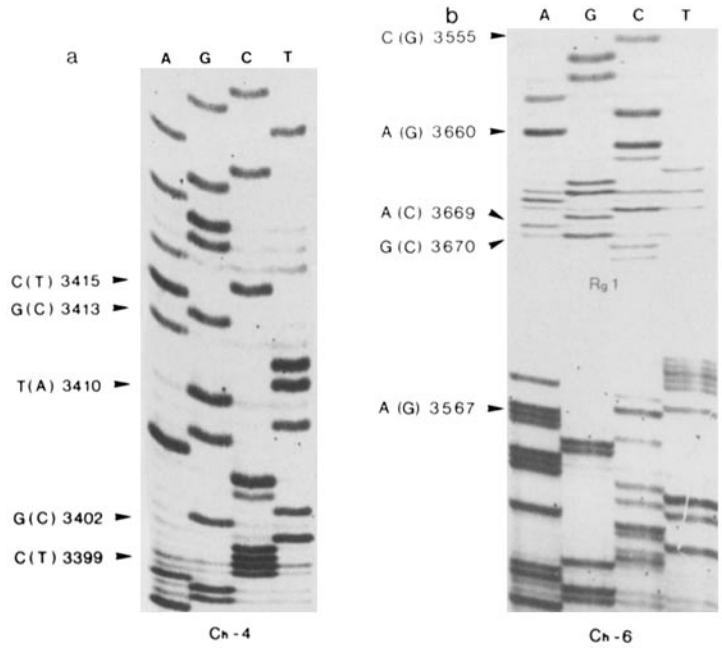

FIGURE 3. Genomic DNA sequence obtained after PCR amplification with PCR primers L3/L4. The DNA was selected from an individual homozygous for the haplotype HLA-*B44*, *C4A3 BQ0* and without gene deletions. The nucleotides typical for C4A are indicated and the alternative C4B nucleotides are given in brackets. The numbers indicate the respective position within the C4 cDNA sequence.(28). (*a*) The sequence of the isotype-specific region correlated to Ch4/Ch-4 (coding strand). (*b*) *Top*: The sequence correlated to the serological determinant Rg1/Ch1 (non-coding strand). *Bottom*: The sequence correlated to the serological determinant Ch6/Ch-6(Rg2) (coding strand).

obtained by oligonucleotide hybridization and RFLP analysis. No ambiguous nucleotide position was observed that would then indicate the presence of two different isotypes.

The complete haplotype data are summarized in Table II. All six *AQ0* alleles were found on haplotypes with *HLA-Bw60* and a short *C4B* gene (16 kb), as indicated by the 5.4-kb Taq I fragment (Table II A). The DNA analysis has revealed the presence of C4A-specific sequences not detectable at the protein level by SDS PAGE, high voltage gel electrophoresis, and serology. We therefore conclude that these non-deleted genes are *C4A* pseudogenes on these haplotypes. The structural basis of the defect, which is not yet determined, may be due to point mutations or deletions, similar to those already observed in the 3' adjacent *21-OHA* pseudogene (31, 32). These mutations may have occurred either in the coding sequence resulting in a stop codon, or may have affected the sequences controlling the expression of the *C4A* gene.

We have detected only C4A-specific sequences in *BQ0* alleles. All the haplotypes with *BQ0* alleles had long *C4* genes at both C4 loci, as defined by the 7.0 and 6.0 kb Taq I fragments (Table II, B–C). When *C4A* alleles at each locus of one haplotype are *identical*, the individual gene products cannot be distinguished, e.g., *C4A3, (A3)*

BQ0 (Table II B), and expression of the second locus cannot be proven. However, the expression of "converted" locus II genes is detectable when the *C4A* allele is *different* from that expressed by the accompanying *C4A* gene at locus I (5, 21, 22). If expression of the converted gene can be demonstrated conclusively, then it can no longer be referred to as null allele. 6 of the 14 *BQ0* haplotypes only expressing the C4A3 allotype carry the alleles for *HLA-B44*, *DR4* (or *DR5*, *DRw6*) (Table II B). A single similar haplotype has been detected previously by using genomic RFLP analysis (5). The expression of two different *C4A* alleles on the same haplotype can be identified only by segregation analysis in informative families. In five haplotypes, A3, A2 expression is considered probable and both genes carry C4A-specific sequences except for the Ch6 determinant (Table II C).

Deletions and duplications of *C4* genes leading to haplotypes with a single or three C4 genes have been demonstrated by RFLP studies and some of these may be due to a single unequal crossover event (18, 19). However, gene conversion has been suggested as a mechanism for the aberrant C4A1 (Ch+) and C4B5 (Rg+) allotypes (15, 16), which seems more acceptable than recombination by multiple crossover events. Gene conversion may be expected when two closely linked genes have arisen by duplication and have a high degree of structural homology, as is the case for the *C4A* and *C4B* genes (1, 2, 26). Our data suggest that conversion has occurred only at locus II (C4B). Recently, however, an individual has been described with a haplotype carrying two loci (a long and a short C4 gene) both expressing C4B isotypes (*C4AQ0 B1,5*). This may serve as an example that a complete conversion at locus I (C4A) is also possible, but obviously less common (33). As only six *C4AQ0* haplotypes without *C4A* deletions were available for study and all of these are linked to *HLA-Bw60*, they may have arisen from a single mutation. This could also be the case for the six converted locus II genes carrying C4A-specific sequences, including the Ch-6 sequence and linked to *HLA-B44*. It remains to be shown, however, whether these converted genes truly express the C4A3 allotype. This can be conclusively proven only by cloning and expressing the entire gene in transfected cells, as has been demonstrated for normal human C4 genes (34). Our approach to the study of C4 null alleles allows the rapid analysis of extensively typed individuals to further investigate the molecular basis of C4 deficiency. At the same time the *C4* gene region of the MHC may serve as a model to study the complex genetic mechanism of gene conversion.

Summary

The two genes for the C4A and C4B isotypes of the fourth component of human complement are located in the MHC class III region. Previous studies have demonstrated the unusual expression of *C4* genes in the form of aberrant or duplicated haplotypes. Null alleles of *C4A* or *C4B* (*AQ0* or *BQ0*) have been defined by the absence of gene products and occur at frequencies of 0.1–0.3. However, only some *C4* null alleles are due to gene deletions, the remainder were thought to be nonexpressed genes. We have analyzed the *C4* gene structure of 26 individuals lacking either C4A or C4B protein. The DNA of individuals with apparently nonexpressed *C4* genes was tested for the presence of C4A- and C4B-specific sequences using restriction fragment analysis and isotype-specific oligonucleotide hybridization of DNA amplified

by polymerase chain reaction. All nondeleted *AQ0* alleles had C4A-specific sequences and may thus be described as pseudogenes, whereas the nondeleted *BQ0* alleles had C4A- instead of C4B-specific sequences. Gene conversion is the probable mechanism by which a *C4A* gene is found at the second C4 locus normally occupied by *C4B* genes.

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