

Genetic Sophistication of Human Complement Components *C4A* and *C4B* and *RP-C4-CYP21-TNX* (RCCX) Modules in the Major Histocompatibility Complex

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Human populations are endowed with a sophisticated genetic diversity of complement *C4* and its flanking genes *RP*, *CYP21*, and *TNX* in the RCCX modules of the major histocompatibility complex class III region. We applied definitive techniques to elucidate (a) the complement *C4* polymorphisms in gene sizes, gene numbers, and protein isotypes and (b) their gene orders. Several intriguing features are unraveled, including (1) a trimodular RCCX haplotype with three long *C4* genes expressing *C4A* protein only, (2) two trimodular haplotypes with two long (L) and one short (S) *C4* genes organized in LSL configurations, (3) a quadrimodular haplotype with four *C4* genes organized in a SLSL configuration, and (4) another quadrimodular structure, with four long *C4* genes (LLLL), that has the human leukocyte antigen haplotype that is identical to ancestral haplotype 7.2 in the Japanese population. Long-range PCR and *Pst*AI-RFLP analyses conclusively revealed that the short genes from the LSL and SLSL haplotypes are *C4A*. In four informative families, an astonishingly complex pattern of genetic diversity for RCCX haplotypes with one, two, three and four *C4* genes is demonstrated; each *C4* gene may be long or short, encoding a *C4A* or *C4B* protein. Such diversity may be related to different intrinsic strengths among humans to defend against infections and susceptibilities to autoimmune diseases.

Introduction

Human populations are endowed with a distinct genetic diversity of complement component *C4* that may confer varying intrinsic strengths on different individuals in the defense against infections, but also may confer vulnerabilities for autoimmunity (Porter 1983; Blanchong et al. 2001; Martinez et al. 2001). Since 1969, when Rosenfeld et al. (1969), using the antibody-antigen crossed electrophoresis, illustrated, for the first time, complement *C4* polymorphisms in the hemolytic activities and electrophoretic mobilities, the progress toward understanding the sophisticated genetics of human *C4* has taken a somewhat meandering course. It appears that there are multiple allotypes of *C4* proteins (Mauff et al. 1998). These protein allotypes may be grouped into two classes: the acidic *C4A* (MIM 120810), with lower hemolytic activities, and the basic *C4B* (MIM 120820) with higher hemolytic activities (Awdeh and Alper 1980). There is a wide spectrum of *C4A* and *C4B* protein levels in the population—from

complete absence of *C4A* or *C4B*, more *C4A* than *C4B*, and more *C4B* than *C4A* to equal quantities of *C4A* and *C4B* (Moulds et al. 1990; Rebmann et al. 1992). A single locus with codominant expressions of the fast- and slow-migrating forms of *C4* was first proposed (Teisberg et al. 1976) but was soon replaced by a two-locus model with one *C4A* gene and one *C4B* gene (O'Neill et al. 1978). The differential levels of *C4A* and *C4B* proteins in the plasma were explained by the presence of null or silent alleles in either the *C4A* locus or the *C4B* locus. The model was supported by work from independent laboratories (Awdeh et al. 1979; Roos et al. 1982). However, there are abundant exceptions to a rigid *C4A-C4B*, two-locus model of *C4* genes in the major histocompatibility complex (MHC) class III region on chromosome 6 (Teisberg et al. 1976; Carroll et al. 1984). Family segregation studies showed considerable frequencies of single-locus haplotypes encoding *C4A* or *C4B* proteins and of three-locus haplotypes encoding one *C4A* and two different *C4B* allotypes (Olaisen et al. 1980; Teisberg et al. 1988). There is also mounting evidence for the homoexpression of *C4A* proteins from two consecutive *C4* loci (e.g., *C4A3 A2*) (Raum et al. 1984; Rittner et al. 1984; Schendel et al. 1985). The phenomenon was observed because, in those cases, each *C4* gene encodes a polymorphic variant distinguishable in an allotyping gel (Awdeh and Alper 1980; Sim and Cross 1986).

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Molecular characterization of various *C4A* and *C4B* genes from individuals with defined phenotypes allowed identification of amino acid residues contributed to the differential chemical reactivities of the activated *C4A* and *C4B*, as well as their associated Rg and Ch blood-group antigens (Carroll et al. 1984, 1990; Yu et al. 1986, 1988; Dodds et al. 1996). It enabled the creation of molecular biologic techniques to determine the presence and number of *C4A* and *C4B* genes in the MHC (Chung et al. 2002; Yu et al. 2002) and led to the elucidation of dichotomous features of *C4* and other constituent genes of the *RP-C4-CYP21-TNX* (RCCX) modules (fig. 1). First, there are long and short forms of the *C4* gene, 20.6 and 14.2 kb in size, respectively. The long *C4* gene has an endogenous retrovirus of 6.36 kb integrated into intron 9 (Dangel et al. 1994; Schneider et al. 2001). The size of intron 9 in the short *C4* gene is only 416 bp. Second, a *C4* gene may encode a *C4A* or *C4B* protein; the basis for this variation is five nucleotide substitutions in exon 26, leading to four isotype-specific amino acid residues (i.e., PCPVLD 1101-6 in *C4A* and LSPVIH 1101-6 in *C4B*) (Belt et al. 1985; Yu et al. 1986). In haplotypes with two or more *C4* genes, duplication is discretely modular of 32.7 kb (long [L]) or 26.3 kb (short [S]) including an intact *C4A* or *C4B* gene, a gene for the steroid *CYP21* (MIM 201910) that is frequently the pseudogene *CYP21A*, and partially duplicated gene fragments *TNXA* (MIM 600985) and *RP2* (MIM 604977).

Initial structural characterization for a limited number of clones revealed that the *C4* gene that is linked to factor

B at the 5' region (i.e., located at the telomeric end) is a long gene encoding *C4A*; a short *C4B* gene is present at the centromeric region linked to the functional *CYP21B* gene (Carroll et al. 1984, 1985). However, population studies using an informative or definitive RFLP analysis coupled with phenotypic characterization of *C4A* and *C4B* proteins revealed a great repertoire of length variants for the RCCX modules with different combinations of genotypic features for the RCCX constituents (Blanchong et al. 2000). A dynamic 1-2-3-locus model for *C4* genes is proposed as close to one-third of the chromosomes 6 in the normal white population that has either monomodular RCCX haplotypes with a single copy of *C4* gene or trimodular haplotypes with three copies of *C4* genes in one chromosome. Only 55% of the white MHC haplotypes have the two-locus structures with *C4A-C4B* configurations in the MHC. The remainder have different combinations (or unequal numbers) of *C4A* and *C4B* genes. It is speculated that the diversities in *C4A* and *C4B* protein levels and in functional properties were one of the intrinsic immune responses to defend against a vast variety of bacterial, viral, and other microbial infections. The high frequency of heterozygosity in RCCX length variants is probably a mechanism to achieve or maintain diversities of *C4A* and *C4B* proteins in the population, since it would allow the exchange of genetic information among homologs of the RCCX constituents. However, heterozygous length variants of RCCX modules could also promote unequal crossovers during meiosis, leading to gene deletions, gene duplications, and disease associ-

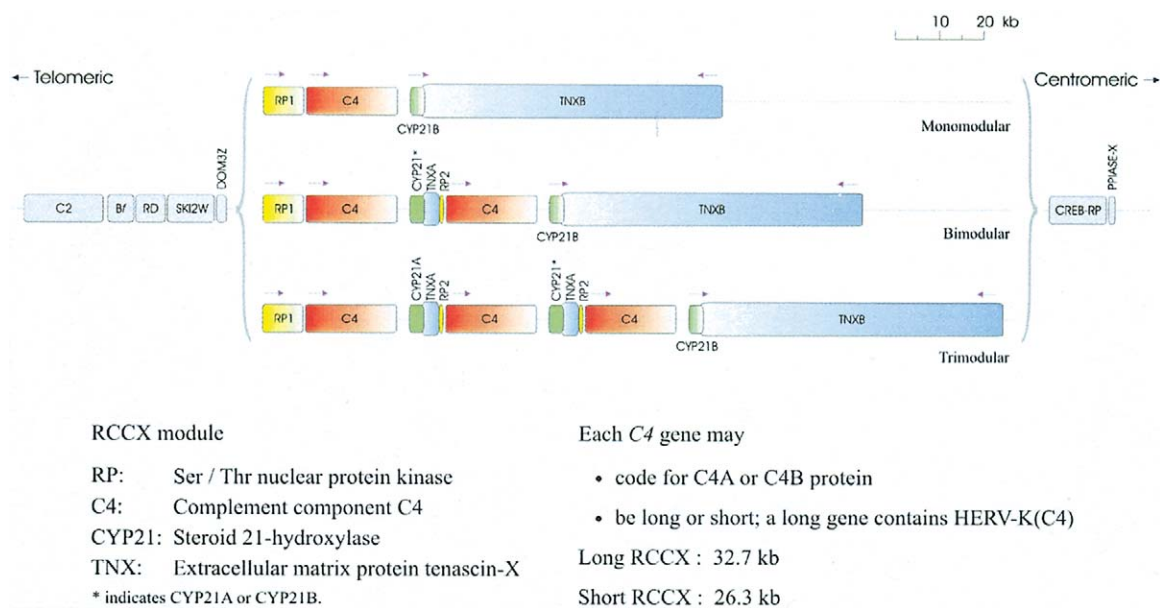


Figure 1 The 1-2-3-locus concept of human *C4* and RCCX modular variations. This map of the human MHC class III region shows the physical length variants among the monomodular, bimodular, and trimodular RCCX structures. The MHC class I genes are located at the telomeric end; the MHC class II genes are located at the centromeric end.

ations (Rupert et al. 1999; Z. Yang et al. 1999; Blanchong et al. 2000, 2001; Jaatinen et al. 2002).

To define the dosage of *C4A* and *C4B* genes together with the other RCCX constituents, we have applied novel, more accurate techniques to directly address the number of *C4* genes present in a diploid genome and in an MHC haplotype. Thus, we further demonstrate the sophistications of *C4A* and *C4B* genetics. New varieties of RCCX modular haplotypes are shown. These include two trimodular structures with LSL configurations, a trimodular LLL haplotype with homoexpression of *C4A*, and two quadrimodular haplotypes, each with four *C4* genes, from pediatric patients with juvenile rheumatoid arthritis (JRA) or with systemic lupus erythematosus (SLE [MIM 152700]).

Material and Methods

Human Subjects, Genomic DNA, and Southern Blot Analysis

Peripheral-blood samples were taken from healthy individuals, patients with SLE, and patients with pauciarticular JRA and relatives after informed consent was obtained, after approval by the Columbus Children's Hospital institutional review board. The isolation of genomic DNA and Southern blot analysis were performed as described elsewhere (Blanchong et al. 2000; Chung et al. 2002).

Labeled-Primer Single-Cycle Polymerization (LSP)–RFLP Analysis of C4A and C4B

A 1.1-kb genomic fragment spanning complement *C4* exons 26–29 was amplified by PCR and was subjected to *PshAI*-LSP RFLP and *XcmI*-LSP RFLP, as described elsewhere (Chung et al. 2002).

Human Leukocyte Antigen (HLA) Typing

HLA class I genes *A*, *B*, *C*, and class II genes *DRB1*, *DRB3/4/5*, *DPB1*, and *DQB1* were typed by sequence-specific primer PCR of genomic DNA, using commercial kits from One Lambda (for HLA *A*, *B*, and *DR*), Pel-Freez (for HLA *C*), and INNO-LiPA (Innogenetics; for HLA *DQB1* and *DPB1*) and standardized protocols.

Complement C4 and C3

Polymorphisms of complement *C4A* and *C4B* proteins were determined by immunofixation of EDTA-plasma after published protocols (Yu et al. 2002). Complement *C3* typing was performed by immunofixation using a method similar to that of *C4*, except that the quantity of plasma was reduced by fivefold. Goat antiserum for human *C3* was purchased from Diasorin.

The concentrations of human *C4* in EDTA-plasma

were determined by a sandwiched-ELISA method. All dilutions were performed using PBS (pH 7.4). In brief, diluted goat anti-human *C4* sera (dilution, 1:300) in PBS was coated on ELISA plate overnight at room temperature. Washing with PBS and treatment with blocking buffer (Pierce) were performed four times after coating, reactions with diluted plasma, primary antibody, and secondary antibody. Plasma samples at 1:4,000 dilution was added to each well and incubated at room temperature for 1.5 h. The primary antibody used was a rabbit antiserum against human *C4* (immunoglobulin G fraction; Sigma; 1:50 dilution). The secondary antibody used was a mouse monoclonal against rabbit immunoglobulin G conjugated with alkaline phosphatase (Sigma; 1:40,000 dilution). After addition of the substrate PNPP (obtained from Pierce) for 1 h, the reaction was terminated by addition of 0.5 M NaOH, and optical density at 405 nm was determined with an ELISA plate reader. A standardization curve of *C4* protein levels was determined using purified *C4* protein (Calbiochem), with quantities varying from 1.56 ng to 100 ng and subjected to the same experimental procedures in the same microtiter plate. *C4* protein level for each plasma sample was assayed twice and each time in triplicate. The validity of the assay was verified with a radial immunodiffusion method using a kit purchased from the Binding Site.

Pulsed-Field Gel Electrophoresis (PFGE) of PmeI- and PacI-Digested Genomic DNA

Genomic DNA in agarose plugs were digested with *PmeI* or *PacI* restriction enzymes and processed as described elsewhere (Chung et al. 2002; Yu et al. 2002). The following conditions were optimized for *PacI* PFGE: gradient, 6 V/cm; calibration factor, 1.4; initial switch time, 1.79 s; final switch time, 12.91 s; run time, 37 h 40 min; included angle, 120°; ramp, linear. The *PmeI*- and *PacI*-PFGE gels were processed by Southern blot; were hybridized with probes specific to *C4d* and to the 3' region of *TNX*, respectively; and were subjected to autoradiography.

Long-Range PCR of the Short C4 Genes and PshAI-RFLP Analysis

Primers E9.5 and E31.3 were designed to anneal to the 5' end of exon 9 and to the 3' end of exon 31 in *C4*, respectively. The sequence for primer E9.5 is 5'-CCC TGG AGA AGC TGA ATA TGG-3' and E31.3 is 5'-CTT CAG GGT TCC TTT GCT GT-3'. A 1 × Premix D with the Failsafe PCR Enzyme Mix (1.25 U) (Epicentre) was used. PCR conditions were as follows: 1 cycle at 94°C for 3 min; 8 cycles at 94°C for 45 s, 66°C for 1 min (decrease 0.5°C/cycle), 72°C for 9 min; 30 cycles at 94°C for 45 s, 60°C for 1 min, 72°C for 9 min; and 1 cycle

at 72°C for 15 min. For the RFLP analysis, one-fifth of the PCR product was digested with *PshAI* (15 U) overnight at 25°C and was resolved by electrophoresis using 1% agarose gel.

Results

Homoexpression of C4A3 from a Trimodular RCCX Haplotype with Three Long C4 Genes

Previous molecular genetic analysis revealed that white female C008 has a total of six *C4* genes in a diploid genome. *PmeI*-PFGE and *TaqI*-RFLP analyses showed that C008 has the LLL/LLL structures for the RCCX modules. In genomic *PshAI* or *PshAI-PvuII* Southern blots hybridized to a *C4d*-specific probe, C008 exhibited an unusual *C4A:C4B* ratio of 5:1 (Chung et al. 2002). Together with *C4*-allotyping data, heterozygous *C4A3 A3 A3/C4A3 A3 B1* haplotypes are predicted. The presence of three *C4A* genes on a chromosome (or haplotype) has never been reported. To confirm this observation, we recruited family members (two parents and two children) of C008 for segregation analysis (fig. 2A).

Consistent results from the *TaqI* and *PshAI* RFLPs and from the *PmeI* PFGE (figs. 2B–2D) indicate that the parents, C8-1 and C8-2, each carry the heterozygous LLL/LL RCCX structures and a total of five *C4* genes. The two children, C8-4 and C8-5, have the heterozygous LLL/LS and LLL/S structures and a total of five and four *C4* genes, respectively. Interestingly, the ratio of *C4A:C4B* as measured in the genomic *PshAI-PvuII* RFLP (fig. 2F), LSP-*PshAI* RFLP, and LSP-*XcmI* RFLP (fig. 2G) for C8-1 was 4:1, whereas that for C8-2 was 3:2. Therefore, it was deduced that C8-1 has haplotypes of *C4A3 A3 A3/C4A3 B1*, and that C8-2 has haplotypes of *C4A3 A3 B1/C4A3 B1*. This suggests that the *C4A3 A3 A3* haplotype of C008 originates from C8-1. The dosage of *C4A* and *C4B* were faithfully reflected in the corresponding *C4* allotypes (fig. 2E). By contrast, the ratio of *C4A:C4B* as determined by the same techniques for C8-4 and C8-5 were 3:2 and 3:1, respectively. Therefore, it was deduced that C8-4 has haplotypes of *C4A3 A3 B1/C4A3 B1* and that C8-5 has haplotypes of *C4A3 A3 A3/C4A3 Q0 C4B1*. In essence, the family segregation study provides informative support for the presence of homoexpression of *C4A3* from a trimodular LLL structure that is present in all three generations: C8-1, C008, and C8-5.

TaqI RFLP revealed that C8-1 has three *CYP21B* and two *CYP21A* genes. On examination of the segregation of *CYP21B* and *CYP21A* (fig. 2B, lane 1), it becomes clear that the nontransmitted haplotype from C8-1 (i.e., bimodular LL) has the unusual configuration with *21B-21B*. It is of interest that, among the six RCCX haplotypes present in the family of C008, there are six different combinations of *C4A*, *C4B*, *CYP21A*, and *CYP21B* genes.

Trimodular RCCX with Two Long and One Short C4 Genes

An RFLP analysis established that individual C007 has heterozygous bimodular and trimodular RCCX structures with a total of four long and one short *C4* genes in her genome. These *C4* genes could be organized as LLL/LS or LLS/LL or LSL/LL. *PmeI*-PFGE analysis of C007 revealed a 146-kb fragment corresponding to the bimodular LL haplotype that is also shared by her two siblings, C7-1 and C7-2 (figs. 3A and 3B, lanes 2 and 3). C007 also has a 172-kb *PmeI* fragment corresponding to a trimodular structure with two long and one short *C4* genes, implying the presence of a novel trimodular RCCX structure that may be either LLS or LSL.

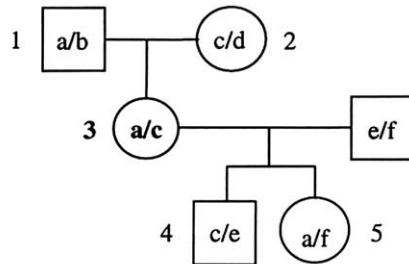
Quadrимodular RCCX with Two Short and Two Long C4 Genes in a Family with JRA

Case report.—J20 is a white female who developed definite pauciarticular-onset JRA at 21 mo of age, when she presented with left-knee synovitis. She has now been followed for 10 years since the onset of her disease. Her JRA has followed a polycyclic pauciarticular course. The only joint that has ever been affected has been her left knee. She has experienced three remissions and three exacerbations of left-knee synovitis since her initial episode. She has been ANA positive but has never been noted to have uveitis. Family history reveals a maternal aunt with adult-onset rheumatoid arthritis.

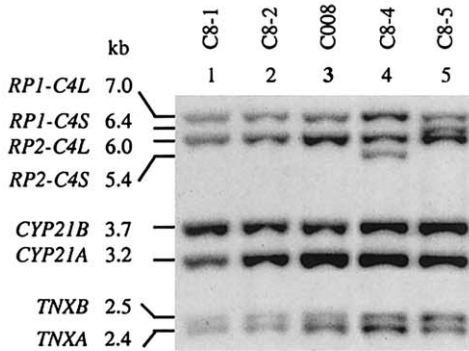
Molecular genetic analysis.—The *TaqI*-RFLP analysis of J20 shows the presence of the 7.0-, 6.4-, 6.0-, and 5.4-kb restriction fragments with respective relative band intensities of 1:1:3:1, indicating the presence of six *C4* genes in total but a puzzled combination of long and short *C4* genes. *PshAI*-RFLP analysis for the relative dosage of *RP2* and *RP1* revealed a 2:1 ratio with six RCCX modules on the two chromosomes (fig. 4B). *PmeI* PFGE clearly shows that one of the chromosomes has the bimodular LL and that the other chromosome has a quadrимodular RCCX structure with two short *C4* genes and two long *C4* genes (fig. 4E). The configuration of these four *C4* genes is undetermined. Since the first RCCX module (i.e., module I) on chromosome 6 always starts with the *RP1* that may be linked to a long *C4* gene (characterized by a 7.0-kb *TaqI* fragment) or to a short *C4* gene (characterized by a 6.4-kb *TaqI* fragment), the quadrимodular RCCX chromosome in J20 starts with *RP1* linked to a short *C4* gene because of the presence of the 6.4-kb *TaqI* fragment. Therefore, the configuration of *C4* genes in the quadrимodular structure has one of the following three combinations: SLL, SL, or SLLS.

One of the parents of J20, J20-1, was recruited for analysis. *PmeI* PFGE revealed that J20-1 and J20 have identical patterns with the quadrимodular and bimodular haplotypes (data not shown). In *TaqI* RFLP, J20-1

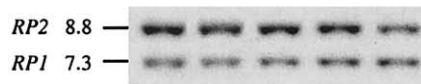
A. Family of C008



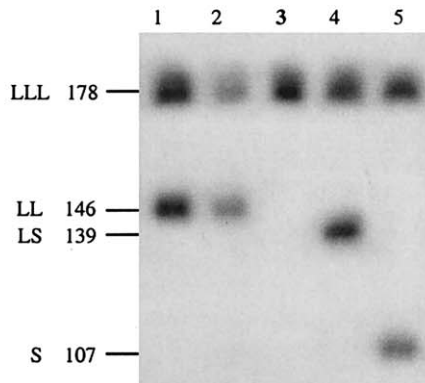
B. RFLP *Taq* I - RCCX



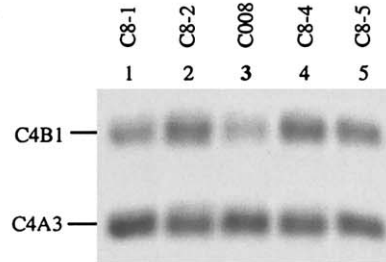
C. RFLP *Psh* A I - RP



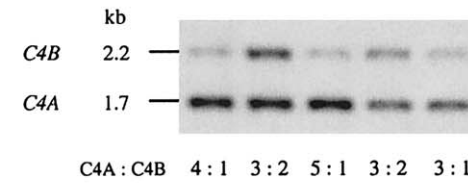
D. PFGE *Pme* I - Bf-TNxB



E. Immunofixation - C4



F. RFLP *Psh* A I + *Pvu* II - C4



G. LSP + RFLP - C4

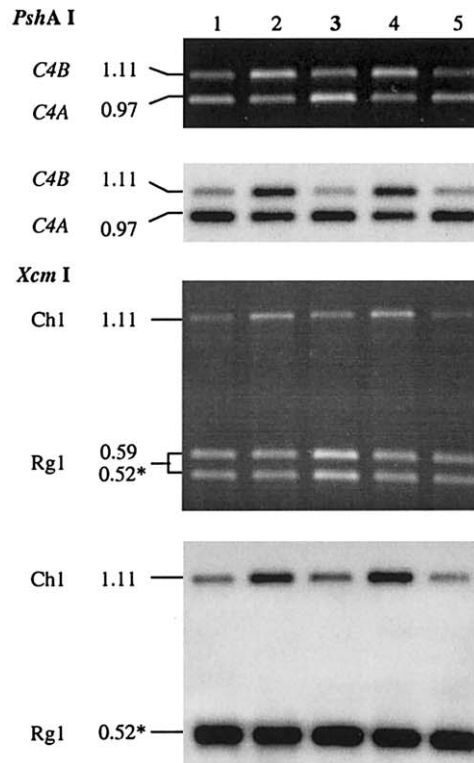


Figure 2 Homoexpression of C4A in a trimodular LLL haplotype from a white family. A, Pedigree of the family C008. B, Genomic *Taq*I RFLP, to show the RCCX modular variations. C, Genomic *Psh*A I-RFLP, to show the number of RCCX modules by determining the relative dosages of *RP2* and *RP1*. D, *Pme*I-PFGE analysis, to show the length variants of RCCX haplotypes. E, Immunofixation, to show the relative quantities of C4A and C4B allotypes. F, *Psh*A I-*Pvu*II RFLP, to show the relative dosage of C4A and C4B genes in each individual. G, LSP-RFLP analysis, to determine the relative dosage of C4A and C4B. The dark-background panels are ethidium bromide-stained pictures that contain artifact results because of heteroduplexes. The light-background pictures are results of autoradiography corresponding to LSP-RFLP images free of heteroduplex effects.

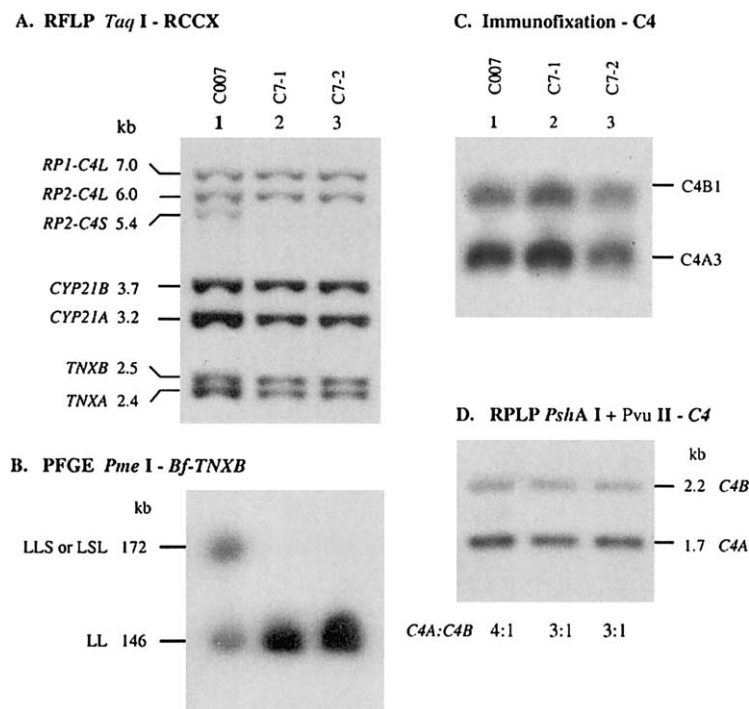


Figure 3 A family with a trimodular RCCX haplotype consisting of two long and one short *C4* genes. *A*, Genomic *Taq*I-RFLP analysis of RCCX modules. *B*, *Pme*I-PFGE analysis, to show haplotypes of RCCX length variants. *C*, Immunofixation of EDTA-plasma, to show the qualitative and quantitative variations of C4A and C4B proteins. *D*, Genomic *Psh*AI-*Pvu*II RFLP, to determine the relative dosage of C4A and C4B.

has the same 1:1:3:1 *RP-C4* gene dosages (fig. 4A). However, there are two notable differences. First, instead of an equal ratio of *CYP21A*:*CYP21B*, as seen in J20, a 2:1 ratio was observed in J20-1, suggesting that the *CYP21* haplotypes for J20 are 21A-21A-21A-21B/21B-21B and that those for J20-1 are 21A-21A-21A-21B/21A-21B. A *Psh*AI-*Pvu*II Southern blot analysis showed that the ratio of C4A:C4B genes in J20 was 2:1 (or 4:2) but showed an astounding 5:1 ratio in J20-1 (fig. 4D, lane 1). Phenotyping experiments revealed the expression of more C4A3 allotype than the C4B1 allotype in the blood plasma of both individuals (fig. 4C). It is therefore predicted that J20 has haplotypes C4A3 A3 A3 B1/A3 B1, whereas J20-1 has haplotypes C4A3 A3 A3 B1/C4A3 A3.

Quadrimodular RCCX with Four Long *C4* Genes in a Family with Pediatric Lupus

Case report.—CS-P4 is an Asian American female who developed definite SLE at age 12 years. Six of the 11 American College of Rheumatology 1997 revised criteria for classification of SLE were fulfilled (Tan et al. 1982; Hochberg 1997). The first manifestation of her lupus was the development of multiple small-vessel vasculitic skin lesions on her fingers and toes (at distal digital tufts, along the sides of her fingers, and within pal-

mar flexor creases on the fingers). Shortly thereafter, she developed a malar rash, polyarthritis, fatigue, a mouth sore on her hard palate, and mild lymphadenopathy.

Initial laboratory testing revealed positive results for the following: antinuclear antibody (1:1,280, speckled pattern), anti-dsDNA antibody (1:40, Crithidia assay), anti-Sm antibody (1:32), anti-RNP antibody (1:64), anti-SS-A antibody (1:128), rheumatoid factor (1:320), anti-Ribosomal P (130, with normal being <20), Coombs, and lupus anticoagulant. Other abnormalities included the following: low C3 (20, with normal being >85), low C4 (<10, with normal being >15), thrombocytopenia (107,000), anemia (10.8), low white-blood-cell counts (3,000), and elevated erythrocyte-sedimentation rate (50, Wintrobe). Her urinalysis results and renal function were normal.

The patient has now been followed for 14 mo since onset of symptoms. Her disease promptly came under control and has remained under good control while she was being treated, cumulatively with corticosteroid, hydroxychloroquine, and methotrexate. No additional lupus features have developed, and she has remained free of renal disease. At her last visit, her C3 and C4 were normal (95 and 23 mg/dl, respectively). The patient's father is Chinese American. He is aware of no family history of lupus or any other autoimmune disease. The

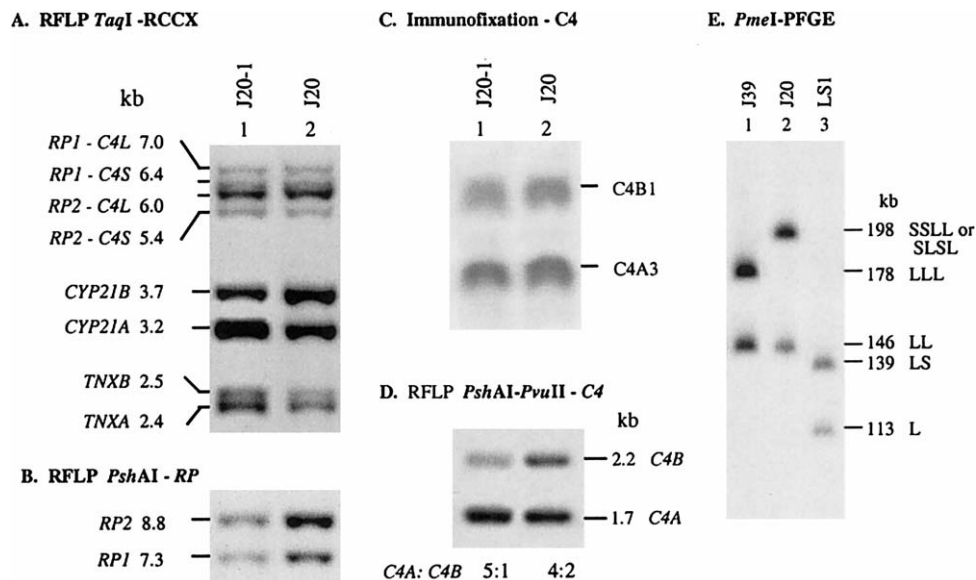


Figure 4 A patient with JRA who had the quadrimodular RCCX haplotype consisting of two short *C4* and two long *C4* genes. *A*, Genomic *TaqI*-RFLP analysis of RCCX modules. *B*, *PshAI* RFLP, to show the relative dosage of *RP2* and *RPI*. *C*, Immunofixation, to determine the *C4A* and *C4B* allotypes. *D*, Genomic *PshAI*-*PvuII* RFLP, to determine the relative gene dosage of *C4A* and *C4B*. *E*, *PmeI* PFGE, to show the quadrimodular RCCX in J20 (lane 2). Lanes 1 and 3 are references for LLL/LL and LS/L RCCX structures, respectively.

patient's mother is Japanese. She knows little of her family history.

Molecular genetic analysis.—A *TaqI*-RFLP analysis of the RCCX gene organizations in CS-P4 revealed a 2-0-3-1 pattern for the restriction fragments corresponding to *RP1*-*C4L*, *RP1*-*C4S*, *RP2*-*C4L*, and *RP2*-*C4S*, respectively. The band intensity for *CYP21B* is half that of *CYP21A*, as is the band intensity of *TNXB* to *TNXA* (fig. 5A, lane 1). These data suggested the presence of six *C4* genes or RCCX modules in this patient, among them four *C4A* and two *C4B* genes, as their corresponding *PshAI*-*PvuII* restriction fragments have a ratio of 4:2 (fig. 5D). The blood sample was taken from the patient during the lupus disease flare stage, when her *C4A* and *C4B* proteins dropped to an almost undetectable level (fig. 5C, lane 1), despite the presence of a considerable amount of complement C3 (fig. 5C, *anti*-C3). A fresh blood sample was taken 6 mo after her disease flare. The *C4* allotyping experiment showed the presence of *C4A3*, B1, and B2 (fig. 5C, *anti*-*C4*, lane 1A). A sandwich ELISA using goat anti-human *C4* as capture antibodies and rabbit anti-human *C4* as primary antibodies confirmed a very low *C4* concentration of 7.4 mg/dl during the relapse, and a partial recovery to 19.7 mg/dl during remission.

Analyses of the RCCX modular structures of CS-P4 revealed a quadrimodular structure with four long *C4* genes (LLLL) in one chromosome and a bimodular structure (LS) with two *C4* genes in the other chromosome, as suggested by the presence of the 210-kb and the 139-kb *PmeI* fragments (fig. 5B, lane 1), respectively. A

PshAI-*PvuII*-RFLP analysis further suggested that CS-P4 has four *C4A* and two *C4B* genes (fig. 5D, lane 1).

The quadrimodular LLLL structure is inherited from the mother (CS-M4), who also has the 210-kb *PmeI* fragment (fig. 5B, lane 2). Unexpectedly, CS-M4 has a 172-kb *PmeI* fragment corresponding to a trimodular LLS or LSL, as in C007 (see the "Trimodular RCCX with Two Long and One Short *C4* Genes" subsection, above). In other words, CS-M4 has a total of seven *C4* genes or RCCX modules. Among them are probably five *C4A* genes (encoding *C4A3*) and two *C4B* genes (encoding *C4B1*) (figs. 5C and 5D, lanes 2). The combined data from *TaqI*-RFLP, *PmeI*-PFGE, *PshAI*-*PvuII*-RFLP, and allotyping experiments showed that the patient's father, CS-F4, has the homozygous bimodular LS/LS structures that encode *C4A3* and *C4B2*.

Configurations of Long and Short *C4* Genes in Trimodular and Quadrimodular RCCX Haplotypes

It has been established that C007 and CS-M4 each have a trimodular RCCX structure that is either LLS or LSL, and J20 has a quadrimodular structure that may be SSSL, SLSL, or SLLS. We further seek to resolve the configuration of the RCCX structures by determining whether a long or short *C4* gene is located immediately upstream from *TNXB*. Deliberate analyses of rare restriction sites in the centromeric end of the HLA class III region (for accession numbers, see the Entrez Nucleotide Web site) revealed the presence of one *PacI* re-

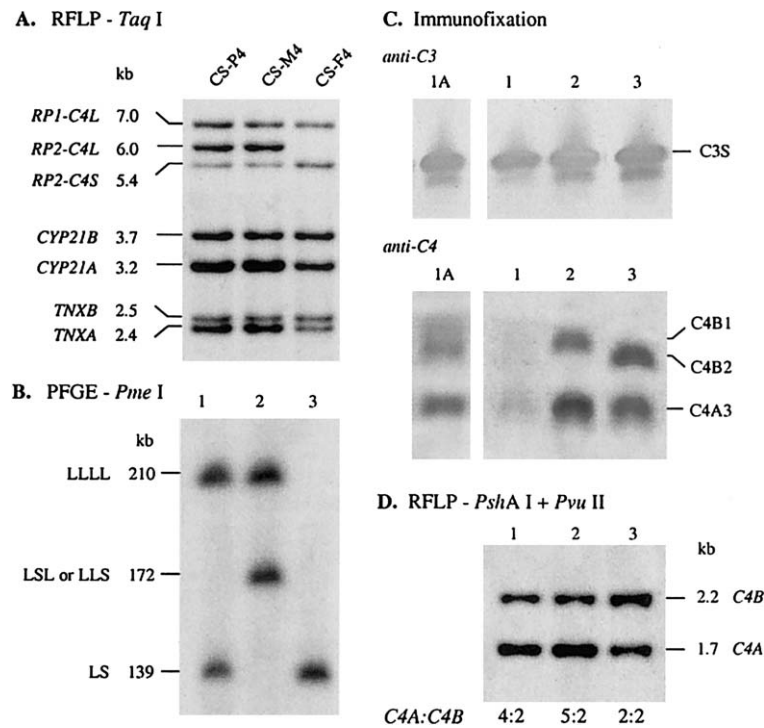


Figure 5 Quadrimodular and trimodular RCCX structures in an Asian family with a pediatric patient with SLE. *A*, Genomic *TaqI*-RFLP analysis of RCCX modules. *B*, *PmeI* PFGE of the length variants in RCCX haplotypes. *C*, Immunofixation of human complement C3 (*anti-C3*) and C4 (*anti-C4*). The sample from lane 1A was taken from the pediatric patient with SLE (CS-P4) while her disease was in remission; the sample from lane 1 was taken from the patient in disease flare. Lanes 2 and 3 were samples from CS-M4 and CS-F4, respectively. *D*, Genomic *PshAI-PvuII* RFLP, to determine the relative dosage of *C4A* and *C4B*.

striction site in *TNXB*, one site in the endogenous retrovirus HERV-K(C4) in each long *C4* gene, and none in *CYP21A/CYP21B* and *RP1/RP2*. The next *PacI* restriction site telomeric to RCCX is located at the intergenic region between complement *C2* and novel gene *G10* (MHC Sequencing Consortium 1999; Milner and Campbell 2001).

Figure 6 shows results of a *PacI*-PFGE analysis, performed using a *TNX* probe for hybridization, of C007 and siblings, CS-P4 and parents, and J20. Lanes 1, 5, and 7 respectively show that C007, CS-M4, and J20 each has a 58-kb restriction fragment representing the presence of three linked *C4* genes with a configuration of LSL, a 48-kb restriction fragment representing a long *C4* gene linked to *TNXB* (*C4L-TNXB*), and a 32.7-kb fragment corresponding to a long *C4* gene linked to another long *C4* gene. In addition, J20 has a 110-kb fragment corresponding to a *PacI* fragment spanning from the intergenic region of *G10* and *C2* to *RP1-C4S-CYP21A-TNXA-RP2-C4L*. From these observations, it was deduced that C007 has a trimodular LSL and a bimodular LL, that CS-M4 has the quadrimodular LLLL and trimodular LSL (lane 5), and that J20 has the quadrimodular SLSL and the bimodular LL (lane 7). The *PacI* restriction patterns in C7-1 (lane 2), C7-2 (lane 3), CS-

P4 (lane 4), and CS-F4 (lane 4) are consistent with previous assignments of RCCX structures.

The Short Genes in LSL and SLSL Haplotypes Are C4A Genes

Although C007 (LSL/LL) and CS-M4 (LLLL/LSL) have *C4* gene dosages of five and seven, respectively, there is only one short *C4* gene in each of their genomes. The quadrimodular RCCX haplotype SLSL in J20 consists of two short *C4* genes, and the homologous chromosome has bimodular LL. For determination of whether the short *C4* genes in the unusual RCCX structures are encoding *C4A* or *C4B* proteins, specific genomic DNA fragments containing the short intron 9—that is, without the endogenous retrovirus HERV-K(C4)—and the *C4A/C4B* isotypic sequences at exon 26 were amplified by PCR, using the primer set E9.5 and E31.3 (fig. 6C). A 7.0-kb genomic DNA fragment spanning exons 9–31 of the short *C4* gene was produced from each of these three individuals (fig. 6D, lanes 2–4). A positive control from an individual with homozygous S/S that encodes C4B1 allotype was included (fig. 6D, lane 1). Aliquots of these PCR fragments were digested with *PshAI* (fig. 6D, lanes 5–8) and were subjected to Southern blot analysis using a *C4A*

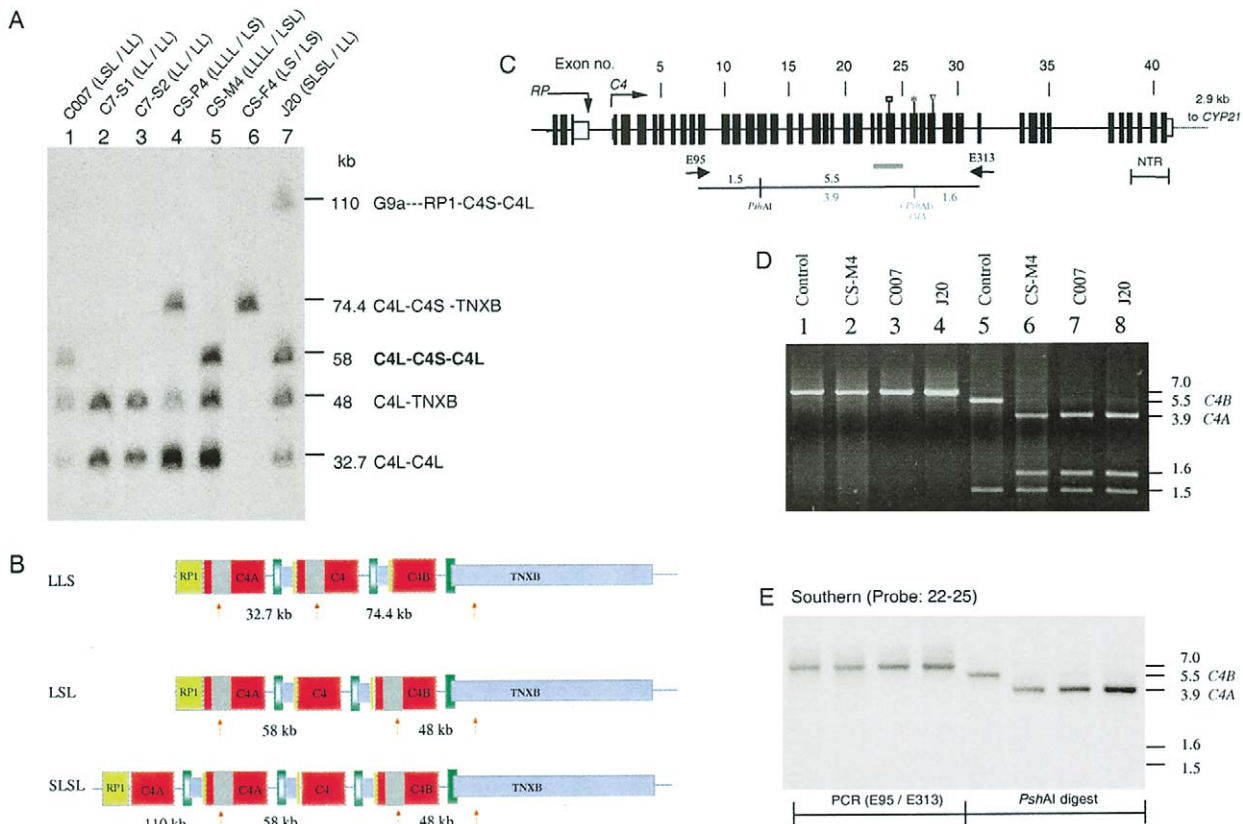


Figure 6 Determining the configurations of long and short C4A and C4B genes in multi-modular RCCX structures. *A*, *PacI*-PFGE analysis of long or short C4 genes linked to TNXA and TNXB. *B*, A scheme illustrating the locations (upward arrows) and size of the *PacI* restriction fragments containing the C4 and TNX in three different length variants. *C*, Exon-intron structure of a short C4 gene and PCR strategy to amplify the genomic region between exons 9 and 31. E9.5 and E31.3 are primers used for amplification of the 7.0-kb genomic fragment for the short C4 gene. The *PshAI* restriction maps for the C4A and C4B genes are shown in red and blue, respectively. *D*, Restriction analysis of the PCR fragments from four different individuals with short C4. Lanes 1–4 are undigested PCR products of E9.5–E31.3. Lanes 5–8 are digested with *PshAI*. Lanes 1 and 5 are from an individual with homozygous, monomodular short C4B genes. *E*, Southern blot analysis of the genomic fragments in panel D, using a C4d exons 22–25 probe.

probe that spans exons 22–25. In the amplified 7-kb DNA fragment, there is a *PshAI* site, in exon 14, that is common to the C4A and C4B genes, and there is an additional site, in exon 26, that is specific to C4A (fig. 6C). The probe hybridized to the 7-kb undigested PCR fragments (fig. 6E, lanes 1–4), the 5.5-kb fragment characteristic of C4B (fig. 6E, lane 5), and the 3.9-kb fragment specific to C4A in C007, CS-M4, and J20 (fig. 6E, lanes 6–8). Therefore, the short genes in LSL haplotypes of C007 and CS-M4 and in SLSL haplotypes of J20 are C4A genes. Results of C4-allotyping experiments indicated that these C4A genes encode C4A3.

The HLA Haplotypes with the Unusual RCCX Structures

The class I and class II alleles of the MHC (i.e., HLA) in the four described families were determined and segregated into haplotypes. The results are shown in table 1 and are categorized as follows:

Monomodular S RCCX.—The monomodular S structure with a single short C4B gene present in C8-5 is typed as having HLA A*01 C*07 B*08, in the class I region, and DRB1*03 DQB1*02 DPB1*0201, in the class II region.

Homoexpression of CYP21B.—Two bimodular LL structures are shown to have the infrequent CYP21B-CYP21B configuration. The first is present in C8-1, with HLA A*32 C*05 B*15(62) DRB1*04 DQB1*0301 DPB1*0401. The second is present in J20, with HLA haplotype A*68 C*04 B*35 DRB1*13 DQB1*0603 DPB1*1901.

Homoexpression of C4A.—Bimodular LL structures with homoexpression of C4A3 (i.e., C4A3 A3) is detected in two haplotypes. The first is in C007 who has HLA A*2 C*05 B*44 DRB1*13 DQB1*0603 DPB1*0401. The second is present in J20-1 who has HLA haplotype A*02 C*03 B*15(62) DRB1*13 DQB1*(0603 or 0607; not determined) DPB1*0401. The trimodular LLL struc-

Table 1

HLA Haplotypes in Families with Trimodular and Quadrimodular RCCX Structures

FAMILY, HAPLOTYPE, AND MHC CLASS	Loci				REMARKS
	1st	2nd	3rd	4th	
Family C008: ^a					
<i>a:</i>					
I—HLA	A*2	C*0303	B*15(62)		
III—C4	A3	A3	A3		LLL
III—CYP	21A	21A	21B		
II—HLA	DRB1*13	DQB1*0603	DPB1*0401		DR52 group
<i>b:</i>					
I—HLA	A*32	C*05	B*15(62)		
III—C4	A3	B1			LL
III—CYP	21B	21B			
II—HLA	DRB1*04	DQB1*0301	DPB1*0401		DR53 group
<i>c:</i>					
I—HLA	A*11	C*15	B*51		
III—C4	A3	A3	B1		LLL
III—CYP	21A	21A	21B		
II—HLA	DRB1*01	DQB1*0501	DPB1*0501		DR1 group
<i>d:</i>					
I—HLA	A*03	C*07	B*07		
III—C4	A3	B1			LL
III—CYP	21A	21B			
II—HLA	DRB1*15	DQB1*0602	DPB1*0401		DR51 group
<i>e:</i>					
I—HLA	A*31	C*08	B*65		
III—C4	A3	B1			LS
III—CYP	21A	21B			
II—HLA	DRB1*07	DQB1*02	DPB1*0301		DR53 group
<i>f:</i>					
I—HLA	A*01	C*07	B*08		
III—C4	B1				S
III—CYP	21B				
II—HLA	DRB1*03	DQB1*02	DPB1*0201		DR52 group
Family C007: ^b					
<i>a:</i>					
I—HLA	A*2	C*05	B*44		
III—C4	A3	A3			LL
III—CYP	21A	21B			
II—HLA	DRB1*13	DQB1*0603	DPB1*0401		DR52 group
<i>b:</i>					
<i>c:</i>					
I—HLA	A*11	C*0303	B*55		
III—C4	A3	A3	B1		LSL
III—CYP	21A	21A	21B		
II—HLA	DRB1*13	DQB1*0607	DPB1*0401		DR52 group
<i>d:</i>					
I—HLA	A*68	C*15	B*51		
III—C4	A3	B1			LL
III—CYP	21A	21B			
II—HLA	DRB1*04	DQB1*0302	DPB1*0301		DR53 group

(continued)

Table 1**HLA Haplotypes in Families with Trimodular and Quadrimodular RCCX Structures**

FAMILY, HAPLOTYPE, AND MHC CLASS	Loci				REMARKS
	1st	2nd	3rd	4th	
Family JRA 20: ^d					
<i>a:</i>					
I—HLA	A*68	C*04	B*35		
III—C4	A3	B1			LL
III—CYP	21B	21B			
II—HLA	DRB1*13	DQB1*0603 or DQB1*0607 ^c	DPB1*1901		DR52 group
<i>b:</i>					
<i>c:</i>					
I—HLA	A*31	C*0303	B*15(62)		
III—C4	A3	A3	A3	B1	SLSL
III—CYP	21A	21A	21A	21B	
II—HLA	DRB1*13	DQB1*0603 or DQB1*0607 ^c	DPB1*0401		DR52 group
<i>d:</i>					
I—HLA	A*02	C*03	B*15(62)		
III—C4	A3	A3			LL
III—CYP	21A	21B			
II—HLA	DRB1*13	DQB1*0603 or DQB1*0607 ^c	DPB1*0401		DR52 group
Family CS4: ^f					
<i>a:</i>					
I—HLA	A*11	C*01	B*46		
III—C4	A3	B2			LS
III—CYP	21A	21B			
II—HLA	DRB1*09	DQB1*0303	DPB1*0501		DR53 group
<i>b:</i>					
I—HLA	A*11	C*0304	B*13		
III—C4	A3	B2			LS
III—CYP	21A	21B			
II—HLA	DRB1*12	DQB1*0301	DPB1*1301		DR52 group
<i>c:</i>					
I—HLA	A*24	C*07	B*07		
III—C4	A3	A3	A3	B1	LLLL
III—CYP	21A	21A	21A	21B	
II—HLA	DRB1*01	DQB1*0501	DPB1*0402		DR1 group
<i>d:</i>					
I—HLA	A*02	C*03	B*61		
III—C4	A3	A3	B1		LSL
III—CYP	21A	21A	21B		
II—HLA	DRB1*11	DQB1*0301	DPB1*0201		DR52 group

^a C8-1: *a/b*; C8-2: *c/d*; C008: *a/c*; C8-4: *c/e*; C8-5: *a/f*.

^b C007: *a/c*; C7-1: *a/d*; C7-2: *a/d*.

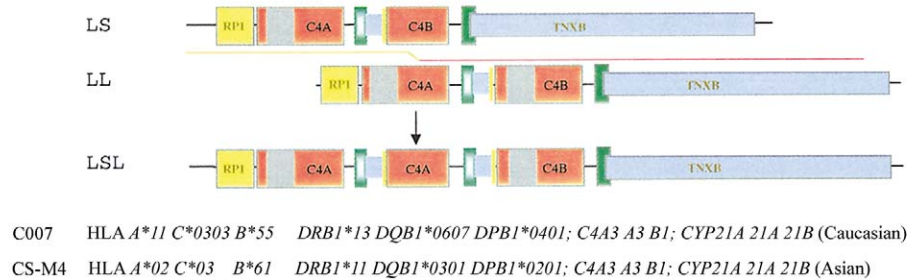
^c Not available.

^d J20: *a/c*; J20-1: *c/d*.

^e Not determined.

^f CS-F4: *a/b*; CS-M4: *c/d*; CS-P4: *a/c*.

A Recombination between LS and LL to generate LSL



B Recombination between SS and LSL to generate SLSL

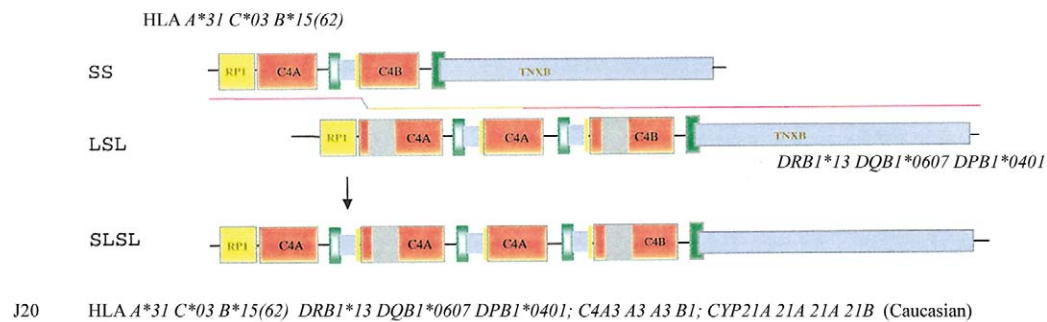


Figure 7 Mechanistic models to generate LSL (A) and SLSL (B) modules of RCCX by unequal crossovers

ture with homoexpression of C4A3 in C008 is present between HLA A*2 C*0303 B*15(62) and DRB1*13 DQB1*0603 DPB1*0401.

Trimodular structures expressing C4A3 A3 B1.—Three haplotypes are shown that express two C4As and one C4B. The first is present in a trimodular LLL of C008 and is located between HLA A*11 C*15 B*51 and DRB1*01 DQB1*0501 DPB1*0501. The second is present in C007, who has an LSL structure in HLA A*11 C*0303 B*55 and DRB1*13 DQB1*0607 DPB1*0401. The third is in CS-M4, who also has an LSL structure, but her HLA is A*02 C*0303 B*61 and DRB1*11 DQB1*0301 DPB1*0201.

Quadrimeric RCCX haplotypes.—The quadrimeric LLLL in the pediatric patient with SLE CS-P4, encoding C4A3 A3 A3 B1, is present between HLA A*24 C*07 B*07 and DRB1*01 DQB1*0501 DPB1*0402. The quadrimeric SLSL structure in J20, encoding C4A3 A3 A3 B1, has HLA haplotype A*31 C*0303 B*15(62) DRB1*13 DQB1*0607 DPB1*0401.

Discussion

Studies on the molecular genetics of complement C4A and complement C4B gene dosages and RCCX modular variations continue to yield surprises. Here, we

elucidated novel haplotypes with three and four RCCX modules that contain long and short C4 genes. In an unaffected white individual (C007) with HLA haplotype A*11 C*0303 B*55 DRB1*13 DQB1*0607 DPB1*0401 and in an unaffected Asian individual (CS-M4) with HLA haplotype A*02 C*03 B*61 DRB1*11 DQB1*0301 DPB1*0201, trimodular RCCX haplotypes with two long and one short C4 genes were demonstrated by *TaqI* RFLP and *PmeI* PFGE. *PacI* PFGE further shows that the configuration of the C4 genes is LSL, with respect to *RP1* at the telomeric end and *TNXB* at the centromeric end. Long-range PCR amplification of the single short C4 genes in C007 and in CS-M4 and the subsequent *PshAI*-RFLP analysis definitively showed that the short gene (in the middle) encodes C4A. Thus, the order of the three C4 genes in each case is probably C4A3, A3, and B1.

By similar approaches, a peculiar quadrimeric RCCX structure with two short and two long C4 genes with a configuration of SLSL is elucidated in a white pediatric patient with pauciarticular JRA (J20). *PshAI*-*PvuII* RFLP showed that three of these C4 genes encode C4A and that the remaining gene encodes C4B. This SLSL structure is located between HLA A*31 C*03 B*15(62) and DRB1*13 DQB1*0607 DPB1*0401. It is noteworthy that the haplotype of the centromeric

HLA class II alleles in this quadrimodular SLSL structure is identical to that of the trimodular LSL structure in C007. A model to illustrate the generation of LSL and SLSL is presented in figure 7. We suggest that the trimodular LSL haplotype could have derived from an unequal crossover between a bimodular LS and a bimodular LL (fig. 7A). Another unequal crossover between the trimodular LSL and a bimodular SS would give rise to a quadrimodular SLSL and a monomodular S (fig. 7B).

A different quadrimodular RCCX haplotype with LLLL structure containing three *C4A3* genes and one *C4B1* gene was detected in an Asian family with a pediatric patient with SLE. The RCCX modules contain an intact *RP1* gene at one end and the intact *CYP21B-TNXB* genes at the other end. HLA typing and a segregation analysis showed that the LLLL structure is present in the HLA haplotype *A*24 C*07 B*07 DRB1*01 DQB1*0501 DPB1*0402*. This is a relatively common haplotype in the Japanese population, having a frequency of 4.2% (Tokunaga et al. 1985). Tokunaga et al. (1991) described it as ancestral haplotype (AH) 7.2. Their earlier phenotypic and genotypic characterizations were in close agreement with our observation: plasma proteins from individuals with AH 7.2 had a substantially larger quantity of C4A than of C4B; a *TaqI*-RFLP analysis of the *C4* genes yielded much higher intensities of the 6.0-kb fragment corresponding to the *RP2-C4L* compared to the 7.0-kb fragment corresponding to the *RP1-C4L*; and PFGE of *MluI*-digested genomic DNA revealed a genomic fragment ~50 kb larger than that from individuals with two *C4* genes. Therefore, it was suggested—conservatively, in retrospect—that three long *C4* genes were present in AH 7.2 (Tokunaga et al. 1991). The present study definitively shows that AH 7.2 from the Asian patient with SLE has *four* long *C4* genes. In keeping with the ethnicity of AH 7.2, the LLLL RCCX haplotype was inherited from the patient's mother, who immigrated to the United States from Japan. Another HLA haplotype with four repeating units of long *C4* genes and *CYP21A* pseudogenes was discovered in a patient with congenital adrenal hyperplasia (CAH [MIM 201910]) who had HLA haplotype *A31 B51 DR4* (Collier et al. 1989).

Each of the two quadrimodular LLLL structures described above could have been generated by an unequal crossover between a trimodular LLL chromosome and a bimodular LL chromosome. A crossover between LLL and a regular bimodular LL partner with one *CYP21A* gene and one *CYP21B* gene would give rise to the LLLL(*CYP21B*) haplotype; a recombination between LLL and a bimodular LL with two *CYP21A* genes (which is a CAH-carrier haplotype) would generate the LLLL(*CYP21A*) haplotype.

A third unexpected observation from the present study is the homoexpression of C4A3 protein from a

trimodular RCCX structure with three long *C4* genes in a white female. This was unraveled through a combination of precise genotyping experiments to determine the total number and the relative dosage of *C4A* and *C4B* genes, phenotyping experiments to detect the qualitative and quantitative variations of plasma C4A and C4B levels, and family segregation studies. This may not be the only case for the homoexpression of C4A proteins from a trimodular LLL haplotype, since we noticed a similar pattern in a published study of duplicated *C4A* genes in the French family *St* (Giles et al. 1987). On the basis of the genotyping data from *TaqI*-RFLP analysis, phenotypic analysis of C4A and C4B allotypes and α -chains of C4A and C4B proteins, and hemolytic activities of the C4A and C4B proteins, it is clear to us that the haplotype with HLA *A24 B18 DR1* in the *St* family consists of an LLL structure that encodes C4A3, A3, and A2, although the specific order of these three *C4* genes could not be deduced.

The phenomena described above reveal the plasticity of the RCCX length variants, which leads to frequent *C4A* and *C4B* gene-dosage variation in human populations. Just from the 13 members in four families, we have demonstrated the magnitude of genetic diversities in *C4* and in RCCX. We observed the homoexpression of C4A from bimodular LL (haplotype *a*, C007's family; haplotype *d*, J20's family) and trimodular LLL (haplotype *a*, C008's family), the presence of LSL in a white individual (haplotype *c*, C007's family) and in Asian American individuals (haplotype *d*, CS-P4's family), and the presence of *CYP21B-CYP21B* in bimodular LL from two different HLA haplotypes (haplotype *a*, J20's family; haplotype *b*, C008's family). Although it is well established that deficiency of *CYP21B* is the main cause of CAH (White and Speiser 2000), it is not clear whether high dosage of *CYP21B* has any influence on the production of corticoids and sex steroids in an individual. In other words, we have elucidated haplotypes consisting of one, two, three, and four *C4* genes, with different combinations of long and short *C4* genes encoding C4A or C4B, and we have observed the variation in the dosage of pseudogene *CYP21A* and functional gene *CYP21B*. The *C4A* and *C4B* gene-dosage variation is a main contributing factor for a wide range of C4A and C4B protein levels in the circulation among different individuals. A study of 125 white individuals has shown a significant correlation between *C4* gene dosages and serum C4 levels and hemolytic activities (Y. Yang et al. 2001).

The sophistication in human complement *C4* genetic diversity demands special attention to data interpretation when studying disease associations with C4A or C4B deficiencies and protein polymorphisms. Of particular interest is the presence of six *C4* genes in the pediatric patient with lupus (CS-P4) and in the patient with JRA (J20). It has been well documented that com-

plete deficiency of C4A and C4B or deficiency of C4A is an important risk factor of SLE (Rupert et al. 2002). It is not clear, however, what role high C4 gene dosage plays in the disease pathogenesis. One possibility is that high C4 gene dosage could lead to the production of larger quantities of C4 proteins at local tissues during an inflammatory process that could exacerbate complement-mediated tissue injuries.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

Entrez Nucleotide, <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide> (for C4A [accession number M59816-U07856-M58915], long C4B gene [accession number AF019413], short C4B gene [accession numbers AL049547 and U24578], RP1 or STK19 [accession numbers L26260 and L26261], RP2 [accession numbers L26262 and L26263], CYP21B [accession numbers M26856, M12792, M13936, and AF77974], CYP21A [accession numbers M26857, M12793, and M13935], TNXB [accession number U89337], and TNXA [accession numbers L26263 and U24488])

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for C4A [MIM 120810], C4B [MIM 120820], CYP21 and CAH [MIM 201910], TNX [MIM 600985], RP1 or STK19 [MIM 604977], and SLE [MIM 152700])

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