Deficiency of Human Complement Protein C4 Due to Identical Frameshift Mutations in the C4A and C4B Genes

Marja-Liisa Lokki, Antonella Circolo, Pirkko Ahokas, Kristi L. Rupert, C. Yung Yu and Harvey R. Colten

*J Immunol* 1999; 162:3687-3693; http://www.jimmunol.org/content/162/6/3687

References

This article cites 45 articles, 10 of which you can access for free at: http://www.jimmunol.org/content/162/6/3687.full#ref-list-1

**Why The JI? Submit online.**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Deficiency of Human Complement Protein C4 Due to Identical Frameshift Mutations in the C4A and C4B Genes\textsuperscript{1,2}

Marja-Liisa Lokki,\textsuperscript{*+} Antonella Circolo,\textsuperscript{*,†} Pirkko Ahokas,\textsuperscript{§} Kristi L. Rupert,\textsuperscript{¶} C. Yung Yu,\textsuperscript{¶} and Harvey R. Colten\textsuperscript{3,*}

The complement protein C4, encoded by two genes (C4A and C4B) on chromosome 6p, is the most polymorphic among the MHC III gene products. We investigated the molecular basis of C4 deficiency in a Finnish woman with systemic lupus erythematosus. C-specific mRNA was present at low concentrations in C4-deficient (C4D) patient fibroblasts, but no pro-C4 protein was detected. This defect in C4 expression was specific in that synthesis of two other complement proteins was normal. Analysis of genomic DNA showed that the proposita had both deleted and nonexpressed C4 genes. Each of her nonexpressed genes, a C4A null gene inherited from the mother, a C4A null gene, and a C4B null gene inherited from the father, all contained an identical 2-bp insertion (TC) after nucleotide 5880 in exon 29, providing the first confirmatory proof of the C4B pseudogene. This mutation has been previously found only in C4A null genes. Although the exon 29/30 junction is spliced accurately, this frameshift mutation generates a premature stop at codon 3 in exon 30. These truncated C4A and C4B gene products were confirmed through RT-PCR and sequence analysis. Among the possible genetic mechanisms that produce identical mutations in both genes, the most likely is a mutation in C4A followed by a gene conversion to generate the mutated C4B allele. The Journal of Immunology, 1999, 162: 3687–3693.

\textsuperscript{1}Edward Mallinckrodt Department of Pediatrics, Washington University School of Medicine, St. Louis, MO 63110; \textsuperscript{2}Blood Transfusion Service, Finnish Red Cross, Helsinki, Finland; \textsuperscript{3}Division of Clinical Immunology and Rheumatology, University of Alabama, Birmingham, AL 35294; \textsuperscript{4}Raahe District Hospital, Raahe, Finland; and \textsuperscript{5}Children’s Hospital Research Foundation, Department of Pediatrics, Ohio State Biochemistry Program, and Department of Medical Microbiology and Immunology, Ohio State University, Columbus, OH 43205

Received for publication October 2, 1998. Accepted for publication December 10, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{1}The genomic DNA sequence for the C4d region of the C4BQ0 gene is available from the GenBank database under accession number AF092085.

\textsuperscript{2}This work was supported by grants from the National Institutes of Health (AI24739 and HD17461 to H.R.C.; AR43969 to C.Y.Y.) and the Pittsburgh Supercomputing Center through the National Institutes of Health Center for Research Resources Cooperative Agreement (IP4RR06009 to C.Y.Y.).

\textsuperscript{3}Address correspondence and reprint requests to Dr. Harvey R. Colten, Northwestern University Medical School, 303 East Chicago Ave., Morton Building 4-656, Chicago, IL 60611. E-mail address: colten@nwu.edu

Copyright © 1999 by The American Association of Immunologists
The apparent importance of C4D in the pathophysiology of SLE and perhaps some of the other associated diseases together with relatively few studies that have explored the molecular mechanisms of complete C4D prompted this study of a C4-deficient Finnish woman with SLE. The molecular basis of her complete C4D was elucidated by investigating the biosynthesis of C4 protein, transcription of C4 mRNA, detection of C4A and C4B genes, and nucleotide sequencing to pinpoint the mutations leading to nonexpression of C4A and C4B proteins from both chromosomes.

Materials and Methods

HLA and complement typing

HLA-A, -B, -C, and -DR Ags were assigned by the standard microlymphocytotoxicity test (25). HLA class II typing DRB1, DRB3, DRB5, and DQB1 were also performed by the PCR-based chemiluminescent reverse dot blot method (26). Complement factor B and C4 allelotypes were determined electrophoretically as described by Marcus and Alper (27). Standard methods were used for radial immunodiffusion (28) and total hemolytic complement in gels (Quantiplate, Kallestad, Beaumont, TX).

Cells

Skin fibroblast cell lines were established from the C4-deficient patient, her HLA-identical brother, and a normal individual according to published methods (29, 30). Cells were maintained at 37°C in 5% CO2 in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin.

EBV-infected B cell lines were established from the father and the mother of the C4D patient and maintained at 37°C in 5% CO2 in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% bovine calf serum, 5 mM sodium pyruvate, 2 mM glutamine, 10 U/ml penicillin, 10 g/ml streptomycin, and 1 mM nonessential amino acids. Anticoagulated peripheral blood samples from all the family members were obtained for DNA extraction.

Biosynthetic labeling

Biosynthetic labeling experiments were performed using C4D and normal fibroblasts grown to confluence in 24-multwell tissue culture plates (Corning, Corning, NY) in DMEM containing 10% FCS. Before each experiment the cells were washed twice with warm HBSS (Life Technologies) and incubated for 2 h in DMEM containing [35S]methionine at 250 g/ml (ICN Radiochemical, Irvine, CA; sp. act., 1100 Ci/mmol). DMEM without serum but with 1 mM [35S]methionine was used for the labeling period. In some experiments cells were incubated before labeling with mediators (IFN-γ 1000 U/ml; Amgen, Thousand Oaks, CA) and TNF-α (30 ng/ml; Genentech, South San Francisco, CA)) for 12 h to maximize C4 expression. At the end of the pulse period, the extracellular medium was recovered. The cells were washed twice with warm PBS and lysed by one freeze-thaw cycle in PBS containing 0.5% sodium deoxycholate (Fisher Scientific, Fairlawn, NJ), 1% (w/v) Triton X-100 (Sigma), 10 mM EDTA (Sigma), 2 mM PMSF (Sigma), and 100 μg/ml leupeptin (Boehringer Diagnostics, Somerville, NJ). The lysed cells and extracellular media were clarified by centrifugation, and total protein synthesis was measured by TCA (Sigma) precipitation of 5-μl aliquots of the cell lysate or extracellular medium (31).

Immunoprecipitation and SDS-PAGE

The samples were precleared with Staphylococcus aureus protein A ( Immunoprecipitin, Life Technologies) and then incubated in the presence of goat polyclonal Abs (IgG fraction) to C4, to C3, and to C1INH (Incstar, Scarborough, ME) overnight at 4°C with excess Ab (32). After incubation, excess Staphylococcus protein A was added to capture Ag-Ab complexes. Immunoprecipitates were washed once with PBS containing 0.5% SDS, 1% (w/v) Triton X-100, 0.5% sodium deoxycholate, and 0.5% BSA (ICN Immunologicals, Costa Mesa, CA) and three times with the same buffer without BSA. The immunoprecipitates were subjected to SDS-PAGE under reducing conditions (5% 2-ME) according to the method of Laemmli (33). The m.w. markers were analyzed in parallel lanes and visualized by Coomassie Brilliant Blue R-250 staining. The gels were fixed in a solution of 46% methanol and 8% acetic acid in water for 30 min, rinsed in water, treated with Fluoro-Hance (RPI, Mount Prospect, IL) for 30 min, dried, and exposed with an intensifier screen at ~70°C to Kodak XAR-5 film (Eastman Kodak, Rochester, NY).

DNA isolation and Northern blot analysis

Genomic DNA was extracted from fibroblasts or EBV-transformed lymphoblasts by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation (36). Buffy coats of peripheral blood were collected in cells were digested with proteinase K. After digestion, the cellular proteins were salted out by dehydration and precipitated with a saturated NaCl solution (37). Individual DNA samples of 10 or 15 μg were digested with the appropriate restriction enzymes. The DNA fragments were separated by electrophoresis in an 0.8% agarose gel (ultra-pure agarose, Life Technologies) and transferred to a nylon membrane (Hybond N*, Amersham, Arlington Heights, IL). After hybridization and autoradiography, the DNA samples were denatured, subjected to electrophoresis in 46% methanol and 8% acetic acid in water for 30 min, then three times at 65°C for 15 min for C4, and twice for factor B in 5× SSC/1% SDS and autoradiographed.

DNA probes

The full-length C4 cDNA clone pAT-A and its 476-bp BamH/PvuII fragment specific for the 5′ ends of both C4 genes were used for C4 probing (15, 38). The CYP21-specific probe was a 500-bp Stul/PstI fragment from the 3′ end of the 2.4-kb genomic C4.5 CYP21 clone, provided by Dr. David Chaplin (Washington University School of Medicine, St. Louis, MO). The RP probe was a 1.1-kb fragment corresponding to nucleotides 522-1620 of the RP1 cDNA (13). The TNX probe is a 500-bp fragment corresponding to exons 35–37 of the human TNXA gene (12). The factor B cDNA probe was a 1761-bp PstI fragment isolated from the previously characterized pBFA-28 clone (16). The α-actin probe was an 800-bp PstI-BamHII fragment of a cDNA isolated from chick skeletal muscle (39).

Oligonucleotide synthesis and DNA sequence analysis

All primers used for RT, DNA amplification, and sequencing were synthesized using an automated DNA synthesizer PCR-Mate (model 391, Applied Biosysystems, Foster City, CA) and are shown below. Genomic sequencing was performed using double-stranded templates and a model 373A automated DNA sequencer from Applied Biosystems according to the standard protocol of the taqDyeDeoxy Terminator Cycle Sequencing Kit. Before direct sequencing, all PCR-amplified DNA products were purified on a 1% agarose gel with Whatman DE81 paper (Clifton, NJ) and ethanol precipitation. The region covering genomic DNA from exons 19–29 except for three gaps was sequenced in the father, mother, and progenitor as well as in an unrelated control. Genomic DNA from two other siblings was also used for sequencing some parts of this region. All oligonucleotides were identical with the published C4 genomic sequence (40), except for substitutions that were introduced (oligonucleotides 9 and 13) to generate restriction sites to facilitate cloning. Numbering of nucleotides in the C4 sequence is given in Ref. 40.

Oligonucleotides

The following oligonucleotides were used: 1) 5′-GACACTGGTCCTCCCGAGACTCITCGT-3′, 2) 5′-CGAGGGTCTCCATTGCAATTCGTTG-3′, 3) 5′-GGGCTGCTCTACATTCCATCTCTATG-3′, 4) 5′-GACAAAAGCCCTTCAAGGCTTAAGAG-3′, 5) 5′-GCGGATCCAGTGGTCCATTTGGAAGA-3′, 6) 5′-CCTTGCTCTGCCCAGCATTCACTAGC-3′, 7) 5′-TCATTGGCCCTCACAGAGCCCTTAAGAG-3′, 8) 5′-CGAGGGTCTCCATTGCAATTCGTTG-3′, 9) 5′-CGGCTGCTCTACATTCCATCTCTATG-3′, and 10) 5′-GACACTGGTCCTCCCGAGACTCITCGT-3′.
The patient was a 30-yr-old Finnish woman who developed photosensitivity, a malar rash, polyarthritis, leukopenia, and positive anti-nuclear Ab (1/320), anti-Sm Ab (1/1280), and a weakly positive rheumatoid factor coincident with her first pregnancy and shortly after delivery. In her serum, complement protein C4 was undetectable, and C3 was modestly reduced or at the lower limit of normal (74–111 mg/100 ml). A skin biopsy revealed vasculitis with deposition of IgM and C3. Five years later she developed aphthous ulcers and increased joint symptoms, for which she was treated with prednisone. An exacerbation of mucositis 1 yr later led to an increase in prednisone therapy and subsequent treatment with azathioprine and methotrexate. An ectopic pregnancy and a pregnancy with her second child each precipitated exacerbations of her symptoms. She has had no renal or cardiac disease, no increased susceptibility to infection, hypersensitivity to medications, or central nervous system disease. Family history revealed a younger male sibling (HLA identical) who suffers from photosensitivity. Her children, her parents, and her MHC nonidentical brother are alive and well. Laboratory studies showed undetectable total hemolytic complement (CH100) and no C4 protein by radial immunodiffusion in the patient or her HLA identical brother. Quantitation of C4 and CH100 in the immediate family members and their HLA typing is shown in Fig. 1. In brief, the proposita inherited the HLA A2 B39 Cw7 DRB1*1501 DRB5*0101 DQB1*0602 BS F S, C4AQ0 BQ0; b) A2, B40, Cw3, DRB1*1501 DRB5*0101 DQB1*0602 BS F S, C4AQ0 BQ0; and d) A3, B62, Cw3, DRB1*0401 DRB4*0101, DQB1*0302, BS F S, C4A3 B3.

Amplification of CDN A and genomic DNA

Ten micrograms of total RNA isolated from fibroblasts of C4D and normal individuals was incubated with 10 U of reverse transcriptase at 42°C for 1 h using the buffers and dNTPs supplied in a CDN A synthesis kit (Invitrogen, San Diego, CA). Oligonucleotide 14, antisense to the normal C4 CDN A sequence, was used as a primer in the first-strand synthesis. The CDN A was subsequently amplified by PCR using the first strand as template and oligonucleotide pairs of 9 and 13. These primers were constructed with restriction sites near the 5’ ends, but the PCR product was purified as described above and used for direct sequencing. The first-strand CDN A was initially denatured at 93°C for 1 min with 1 μg of each oligonucleotide in a 100-μl solution containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.1% gelatin, 20 mM dNTPs, and 0.5 U of KlenTaq 1 DNA polymerase (41). Following initial denaturation, the CDN A was amplified by melt denaturation at 93°C for 1 min, annealing at 66°C for 1 min, and polymerization at 72°C for 2 min. Forty cycles of amplification were performed using a programmable Hybrid OmniGene thermal cycle (Labnet, Middlesex, U.K.) followed by a final elongation at 72°C for 5 min. The C4 CDN A was purified and sequenced as outlined above.

Genomic DNA was amplified using 400 ng of DNA and 200 ng of each primer in a primer pair with the other reagents as described above in a “touchdown” protocol (42). The first six cycles were conducted at decreasing annealing temperatures in 2°C steps for two cycles each from 72 to 64°C, followed by 30 cycles using the following conditions: 10 s at 98°C, 1.5 min at 64°C, and 5 min at 72°C. The amplified genomic DNA was purified and sequenced as outlined above.

Cloning and sequencing of the entire C4d genomic regions for the C4B genes

DNA fragments corresponding to the C4d region were amplified with synthetic PCR primers C4E 22.5 and C4E 31.3 using the High Fidelity PCR system (Boehringer Mannheim, Indianapolis, IN). PCR conditions were one cycle at 94°C for 2 min; 10 cycles at 94°C for 15 s, 65°C for 30 s, and 72°C for 1 min; 20 cycles at 94°C for 15 s, 56°C for 30 s, and 72°C for 1 min plus 20-s time extensions; one cycle at 72°C for 7 min; followed by a 4°C dwell cycle. The PCR product was purified (Qiagen, Chatsworth, CA) and cloned into TA cloning vector (Invitrogen). The C4d insert of the clones was amplified again via PCR, and the purified PCR product was restriction digested with NolV to determine the presence of either C4A or C4B. A 467-bp fragment represents C4B, while two fragments (276 and 191 bp) represent C4A (15). Clones representing C4B from both the proposita’s mother and father were sequenced (ABI Prism). Comparison of DNA sequences with national databases was performed by the GCG FASTA program from the Pittsburgh Supercomputing Center. DNA alignments were performed with the SeqManII program (DNASTAR). Synthetic DNA primers for PCR and for cycle sequencing were synthesized by Life Technologies (Grand Island, NY). Oligonucleotide 14, antisense to the normal C4 cDNA, was used in a primer pair with the other reagents as described above in a “touchdown” protocol (42). The first six cycles were conducted at decreasing annealing temperatures in 2°C steps for two cycles each from 72 to 64°C, followed by 30 cycles using the following conditions: 10 s at 98°C, 1.5 min at 64°C, and 5 min at 72°C. Amplification of C4 and CH100 in the immediate family members and their HLA typing is shown in Fig. 1. In brief, the proposita inherited the HLA A2 B39 Cw7 DRB1*1501 DRB5*0101 DQB1*0602 BS F S, C4AQ0 BQ0; b) A2, B40, Cw3, DRB1*1501, DRB5*0101, DQB1*0602, BS F S, C4AQ0 BQ0; and d) A3, B62, Cw3, DRB1*0401, DRB4*0101, DQB1*0302, BS F S, C4A3 B3.

FIGURE 1. The nuclear family. The proposita with SLE and her MHC-identical C4D brother have severe and minimal symptoms, respectively. The serum C4 concentration is in grams/liter/total hemolytic complement activity (in units/ml/normal values, 0.2–0.6/90). Numbers below haplotypes represent C4 concentration/total hemolytic activity, which are in grams per liter and units per milliliter, respectively. The normal values for C4 concentration are between 0.2 and 0.69; the normal values for hemolytic activity are >50. An arrow indicates the proposita. MHC haplotypes are: a) A2, B39, Cw7, DRB1*1501, DRB5*0101, DQB1*0602, BS F S, C4AQ0 BQ0; b) A24, B40, Cw3, DRB1*1302, DRB3*0301, DQB1*0605, BS F S, C4AQ0 B2; c) A2, B40, Cw3, DRB1*1501, DRB5*0101, DQB1*0602, BS F S, C4AQ0 BQ0; and d) A3, B62, Cw3, DRB1*0401, DRB4*0101, DQB1*0302, BS F S, C4A3 B3.

Biogenesis and secretion of C4 in cell culture

To determine whether a defect in regulation of C4 gene expression, C4 synthesis, or secretion accounted for the low to absent C4 protein in sera of the homozygous deficient patient, primary fibroblast cell cultures were established. SDS-PAGE of immunoprecipitates of cell lysates and culture media from [35S]methionine pulse-labeled normal and proposita’s C4D deficient (C4D) fibroblast cultures is shown in Fig. 2. Fibroblasts were incubated with IFN-γ (500 U/ml) and TNF-α (30 ng/ml) to yield maximum C4 expression (24). A polypeptide of approximately 185 kDa, corresponding in size to pro-C4, was detected in lysates, and native C4 subunits (α, ~93 kDa; β, ~78 kDa; γ, ~33 kDa) plus the approximately 125-kDa processing intermediate α-γ fragment were recovered from the culture media in normal fibroblast cultures. Neither pro-C4 nor native C4 protein was detected in IFN-γ- and TNF-α-stimulated C4D fibroblast cultures even after prolonged exposure (10 days) of the autoradiograph. In contrast, both C4D and normal fibroblasts synthesized and secreted comparable amounts of C1
inhibitor, a highly IFN-γ-responsive complement protein, and C3, a protein similar to C4 in size and postsynthetic processing (Fig. 3, A and B). C3 synthesis and secretion were more variable among separate replicate experiments, but the mean C3 expression in C4D cells was not significantly different from normal.

**C4 mRNA expression**

The size and amount of C4 mRNA in normal and C4D IFN-γ- and TNF-α-stimulated fibroblasts were estimated by Northern blot analysis. C4-specific mRNA (5.5 kb) in cytokine-stimulated cells was of similar size in normal and C4D fibroblasts, but the amount of C4 mRNA was markedly reduced in the C4D fibroblasts (Fig. 4). Scanning of replicate Northern blots for C4, factor B, and actin confirmed a selective decrease in C4 mRNA (33:1) and of Bf mRNA of 1.5:1.0 in normal and C4D samples, respectively, when normalized for loading by scanning for mRNA actin.

**Three nonexpressing C4 genes in the patient**

The presence of C4 and its neighboring genes for the RCCX modules in the HLA were investigated to determine the molecular basis of the C4D. Genomic DNAs from the patient and her parents were digested with restriction enzyme TaqI and hybridized to RP, C4, CYP21, and TNX probes. As shown in Fig. 5, the father (lane 3) has the 7.0- and 5.4-kb restriction fragments corresponding to RP1-C4(L) and RP2-C4(S), respectively; the 3.7- and 3.2-kb fragments corresponding to CYP21B and CYP21A, respectively, and the 2.5- and 2.4-kb fragments corresponding to TNXB and TNXA.
genes, respectively. The band intensities for these fragments are similar; therefore, he is homozygous for RP1-C4(L)-CYP21A-TNXA-RP2-C4(S)-CYP21B-TNXB. The C4 allotyping results suggested that he has C4AQ0 C4BQ0 and C4AQ0 C4B2. Therefore, the father has RCCX bimodular (L-S) haplotypes in both chromosomes. Both C4A and C4B genes are present in the C4AQ0 BQ0 chromosome, but these genes are not producing C4 protein. Similarly, the C4AQ0 C4B2 chromosome with the RCCX bimodular (L-S) haplotype is likely to have a C4A pseudogene.

The mother (lane 2) has 7.0- and 6.0-kb TaqI fragments corresponding to RP1-C4(L) and RP2-C4(L), respectively. The 7.0-kb fragment is twice as intense as the 6.0-kb fragment. In addition, her 3.7-kb CYP21B fragment is twice as intense as the 3.2-kb CYP21A fragment, and the 2.5-kb TNXB fragment is more intense than the 2.4-kb TNXA fragment. These results suggest that she is heterozygous for one RCCX bimodular chromosome, RP1-C4(L)-CYP21A-TNXA-RP2-C4(L)-CYP21B-TNXB, and one RCCX monomodular chromosome, RP1-C4(L)-CYP21B-TNXB. Since she has C4A3 B3 on one chromosome and C4AQ0 BQ0 on the other, the monomodular (L) chromosome corresponds to the C4AQ0 C4BQ0 phenotype. One of the C4 genes on this chromosome has been deleted.

For the patient, the 7.0-kb TaqI fragment for RP1-C4(L) is more intense than the 5.4-kb fragment for RP2-C4(S) (lane 4). In addition, the 3.7-kb fragment for CYP21B is more intense than the 3.2-kb CYP21A fragment. She is heterozygous for a RCCX bimodular chromosome RP1-C4(L)-CYP21A-TNXA-RP2-C4(S)-CYP21B-TNXB and one monomodular RCCX chromosome RP1-C4(L)-CYP21B-TNXB. She has two long C4 genes and one short C4 gene. None of these three C4 genes expresses detectable C4 protein. Lane 1 shows the result of a control DNA (T cell line MOLT4) with bimodular RCCX (L-S) haplotypes.

To define and type the C4 genes inherited in this family, a 934-bp genomic fragment spanning from exon 25 to the 5' end of intron 28 that permits differentiating among specific C4 allotypes (8) was amplified from genomic DNA using primers 5 and 11, subjected to digestion with NlaIV and EcoO109, and sequenced. The digests and the patterns characteristic for specific C4 isotypes at each of the C4 genes and sequence analysis are shown in Table I.

To ascertain the molecular mechanism accounting for the non-expressed C4A and C4B genes in the proposita, a 267-bp PCR product spanning the segment from nucleotide 5776 (intron 28) to nucleotide 6043 (exon 29) was generated, and the total PCR product was directly sequenced to search for a mutation previously found in the C4A gene (43). The results (Fig. 6) show a 2-bp (TC) insertion after nucleotide 5880. This insertion leads to a frameshift mutation, a predicted change in amino acid sequence, and a stop signal at codon 3 in exon 30. The HLA-identical brother’s amplified DNA showed the same sequence. Amplified DNA from her haploidentical brother, her father, and her mother showed both normal and mutant sequences. Based on the amplitude of the peaks, the father has the TC insertion in both C4A and C4B genes, whereas the mother has the 2-bp insertion only in her C4A gene. An unrelated control showed only the normal sequence. The proposita showed only the mutated sequence.

To confirm the 2-bp insertion in the C4BQ0 gene, genomic sequences of the C4d region from the father and mother were amplified by PCR, cloned into TA vector, and sequenced. The C4B clones were identified by NlaIV digests (15). Two C4B clones from the father and one from the mother were sequenced to completion. The paternal C4B clones contain the C4B isotypic amino acid sequence LSPVIH at positions 1101–1106, the C4B-associated amino acid sequence ADLR for the Ch1 Ag at positions 1188–1191, and S1157 that is associated with Ch6. Additionally, he has the D1054 that is associated with Ch5 (6). In intron 28, both paternal clones have the nucleotide sequence ggtct that is 14 nucleotides downstream of intron donor site. However, the most distinguishing feature for both paternal C4B clones is the presence of a TC dinucleotide insertion at codon 1213. This is the first definitive proof showing the presence of a 2-bp insertion into a C4B gene. Conversely, the C4B sequence for the maternal clone is identical with the C4B3 sequence published previously, which has the characteristic sequences G1054, LSPVIH 1101–1106, S1157, and ADLR 1188–1191 (5). As expected, the exon 29 sequence is intact, with no insertion.

This 2-bp insertion in exon 29 of C4A was previously reported (43), and the stop codon in the next exon was predicted on the assumption that normal splicing (excision at the normal splice sites) of intron 29 would occur. To directly test this presumption, RNA from the patient’s and normal fibroblasts (stimulated with IFN-γ and TNF-α) was amplified by RT-PCR (primer 14 to generate cDNA and primers 9 and 13 for PCR amplification) and then

---

### Table I. Derived amino acid sequences at the C4d region

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Amino Acid Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td></td>
</tr>
<tr>
<td>a C4AQ0</td>
<td>D P C L D N T V L</td>
</tr>
<tr>
<td>b C4BQ0</td>
<td>D L S I H S T A R</td>
</tr>
<tr>
<td>b C4B2</td>
<td>D L S I H S T A R</td>
</tr>
<tr>
<td>Mother</td>
<td></td>
</tr>
<tr>
<td>c C4AQ0</td>
<td>D P C L D N T V L</td>
</tr>
<tr>
<td>c C4BQ0</td>
<td>(deleted)</td>
</tr>
<tr>
<td>c C4B3</td>
<td>G L S I H S T A R</td>
</tr>
<tr>
<td>Patient</td>
<td></td>
</tr>
<tr>
<td>a C4AQ0</td>
<td>D P C L D N T V L</td>
</tr>
<tr>
<td>a C4BQ0</td>
<td>D L S I H S T A R</td>
</tr>
<tr>
<td>c C4AQ0</td>
<td>D P C L D N T V L</td>
</tr>
<tr>
<td>c C4BQ0</td>
<td>(deleted)</td>
</tr>
</tbody>
</table>

* Complete sequences for this region were obtained but only amino acid residues that vary as a function of allotype are shown (bold, uppercase).

* A2-bp nucleotide insertion at codon 1213 (lowercase).

---

### FIGURE 6. Nucleotide sequence and derived amino acid sequence of the 267-bp PCR products from nucleotide 5776 (intron 28) to 6043 (exon 30) in patient and normal control DNA. The first nucleotide indicated is nucleotide 5867, and first amino acid is codon 1209. Shaded nucleotides indicate the 2-bp insertion; the dotted line indicates nucleotides not shown.
subjected to sequence analysis. These results revealed an exonic sequence identical with that obtained from genomic sequencing and normal splicing between exons 28–30 (data not shown).

Discussion

The C4 genes are expressed in liver (32) and in extrahepatic tissues, including skin fibroblasts (31). C4 mRNA, 5.5 kb in length, programs synthesis of a single-chain preprotein that undergoes extensive posttranslational processing to yield the native three-chain C4 protein. The availability of primary skin fibroblast culture from the C4D patient reported here permitted for the first time an analysis of C4 protein synthesis and secretion in an individual with a genetic deficiency of this protein. Even under tissue culture conditions designed to maximize C4 expression (24), no pro-C4 synthesis was observed in fibroblasts from the C4D patient, whereas synthesis of pro-C4 and secretion of native C4 were readily detectable in control fibroblasts. This defect is selective, since the C4D fibroblasts synthesized and secreted C1 inhibitor and C3 in amounts comparable to those produced by control cells and in published reports (44, 45). The failure to detect pro-C4 could be due to a pretranslational mechanism, a selective defect in pro-C4 translation, or instability of the C4 translation product(s). In part, the decrease in C4 protein expression results from markedly reduced steady state C4 mRNA levels (<3% of normal).

Analysis of the nuclear family established that the patient inherited a nonexpressed long C4A gene from her mother, a nonexpressed long C4A gene, and a nonexpressed short C4B gene from her father. Her maternally derived haplotype, A2; B40; Cw3; DR2 (DRB1*1501, DRB5*0101, DQB1*0602), also contains a deleted C4B gene. C4B deletions have been recognized in association with B40 (46, 47), while the linkage between B40 and DR2 is unusual. Her paternally derived haplotype, A2:B39;Cw7;DR2 (DRB1*1501, DRB5*0101, DQB1*0602), is identical at the DR/DQ loci and differs at the class I loci. A detailed analysis of the basis for lack of expression of the patient’s nondeleted C4 genes was undertaken. This revealed an identical 2-bp insertion in her three nonexpressed C4 genes, which include two C4A genes and one C4B gene. This 2-bp insertion in exon 29 was previously observed by Schneider and colleagues (43) in several C4A null alleles; the majority of them were associated with HLA-B60 and DR6. In their study, based entirely on sequence analysis of genomic DNA (PCR amplified), they predicted that this frameshift mutation would generate a stop codon in the next exon of the C4 transcript derived from this gene. Generation of the stop codon would occur only if splicing of intron 29 was executed at the normal splice sites. Examples of splicing abnormalities, particularly in the context of mutations (including frame shifts) have been reported (48–52). Because C4 is expressed in a readily available tissue (skin fibroblasts), we were able to establish that splicing at the exon 29–30 junction was normal and that a truncated C4B translation product would be generated. This truncated C4 polypeptide was not detected in cell culture, probably because the shortened pro-C4 may be unstable.

Nonsense mutations have been associated with marked decreases in steady state levels of specific mRNA in other instances, such as β-gllobin (53), α1-antitrypsin (54), surfactant protein B (55), cystic fibrosis transmembrane regulator (56), and others (56, 57). Although the basis for this observation is not completely understood (52), a similar mechanism could account for the decreased levels of C4 mRNA observed in this patient (see Fig. 4). Nonetheless, sufficient C4 mRNA was present in cytokine-stimulated fibroblasts to allow nucleotide sequence analysis of RT-PCR products from the homoyzous deficient fibroblasts. In contrast to the earlier study that identified this mutation only in C4AQ0, the 2-bp (TC) insertion was surprisingly also present in the patient’s C4BQ0 nonexpressed genes, giving the first evidence of the molecular basis for C4B pseudogenes. This 2-bp insertion in exon 29 of the nonexpressed C4BQ0 gene could have been acquired from the C4AQ0 gene of the HLA B40-positive haplotype by unequal cross-over or gene conversion-like events. It has been noticed that some of the C4BQ0 phenotypes in the population are attributed to the deletion of the C4B gene (as shown here for the HLA B40 DR2 haplotype) or to the expression of C4A proteins from the two tandem C4 loci as documented in the HLA B44 DR6 haplotype (15). This report shows that the third cause of a C4BQ0 phenotype is due to a 2-bp insertion at codon 1213 of the C4B gene that causes a frameshift mutation.

Acknowledgments

We thank Barbara Hermann for secretarial assistance, Joie Haviland for expert technical assistance, Dr. Rick Wetzel for helpful review of the manuscript, Dr. Juha Kere for generating the EBV cell lines, and Dr. Seppo Meri for quantitating the hemolytic activities.

References


