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Genetics

Homozygous hereditary C3 deficiency due to a partial gene deletion

(*Alu* repeated sequence/homologous recombination/complement 3/immunodeficiency)

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ABSTRACT **The molecular mechanism of *C3* deficiency in an Afrikaans patient with recurrent pyogenic infections was studied. Restriction enzyme analysis showed a gene deletion of 800 base pairs (bp) mapping to the α chain of *C3*. Amplification of genomic DNA, using the PCR, demonstrated that the deletion included exons 22 and 23 of the *C3* gene. Truncated mRNA was shown in an Epstein–Barr virus-transformed B-cell line by PCR amplification of first-strand cDNA. A consequence of this deletion was that the RNA transcribed 3′ to the deletion was out of frame, resulting in formation of a stop codon 19 bp downstream from the deletion. The molecular basis of the deletion was compatible with homologous recombination between two *Alu* sequences located in introns 21 and 23. An unrelated nonconsanguineous relative and two of a sample of 174 Afrikaans-speaking individuals were heterozygous carriers of the same gene deletion. The wide prevalence of this null allele in this population is probably due to the effects of a small founder population. The presence of this deletion in the *C3* gene is not compatible with production of any functional C3, supporting the idea that study of such patients offers a valid model for understanding physiological activities of C3 *in vivo* in humans.**

Analysis of patients with hereditary deficiencies of complement proteins has given insight into the physiological actions *in vivo* of the complement system in host defense against pyogenic infections and disease mediated by immune complexes. Complement protein C3 is the major protein of the complement system in plasma in terms of quantitative expression, at ≈1 g/liter. Cleavage of C3 by C3 convertase enzymes results in release of the anaphylatoxin C3a. The other product of the initial cleavage of C3, C3b, covalently fixed to surfaces, acts as a focus for formation of the C5 convertase enzyme and, together with its further cleavage product, iC3b, is a ligand for complement receptors CR1 and CR3. Absence of C3 is a rare condition in humans and provides the best opportunity for investigating the physiological activities of this protein *in vivo* in humans.

To date 15 patients, who suffered from recurrent pyogenic infections and/or rashes and nephritis (1–10), have been described with inherited homozygous C3 deficiency. Characterization of the full genomic organization of the human *C3* gene (11) allowed us to identify a splice site mutation as the molecular basis for C3 deficiency in a 10-year-old English patient (12). Data have also been reported on the biochemical basis of a genetically determined C3 deficiency in guinea pigs (13). There is evidence for genetic heterogeneity in the molecular basis of C3 deficiency in humans as suggested by the presence of small amounts of circulating C3 in the sera of some patients (4) but not of others (14).

In this paper, we report the molecular basis of C3 deficiency in an Afrikaans patient previously described by Alper and colleagues (1) as homozygous C3 deficient. By Southern blot analysis, we found that the structural alteration of the *C3* null gene was an 800-base-pair (bp) deletion in the α chain including two coding regions (exons 22 and 23). This leads to a disturbance of the reading frame of the C3 mRNA with a stop codon 19 bp downstream from the deletion. DNA sequence analysis revealed that the deletion probably arose from homologous recombination between two *Alu* repeats flanking the deletion, which resulted in generation of a recombined *Alu* element in the deletion sequence. This mutant allele was found to have a gene frequency of 0.0057 in the South African Afrikaans-speaking population.§

MATERIALS AND METHODS

Preparation and Analysis of Genomic DNA. Genomic DNA was isolated from whole blood of the patient, her family, and 174 Afrikaans-speaking white South Africans. DNA (10 μg) was digested to completion with two different restriction enzymes (*Eco*RI and *Sst* I) (GIBCO/Bethesda Research Laboratories) and transferred (15) to Hybond-N membranes (Amersham). The filters were hybridized with a cDNA probe for C3, pC3.11 (16) (kindly provided by G. Fey, Scripps Clinic, La Jolla, CA), which was labeled by the random-primer method (17), washed at high stringency (0.2× standard saline citrate/0.1% SDS at 65°C), and autoradiographed.

mRNA Extraction from B-Lymphoblastoid Cell Line. Mononuclear cells were isolated from 40 ml of peripheral blood of the patient and her family by centrifugation on Lymphoprep (Nycomed, Oslo). B lymphocytes were separated and transformed by Epstein–Barr virus (EBV). EBV-transformed B-cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in the presence or absence of lipopolysaccharide (5 μg/ml). mRNA was extracted by the guanidinium isothiocyanate/cesium chloride method (15).

cDNA Synthesis and PCR Amplification. First-strand cDNA synthesis was performed as described (18) with 2 μg of total mRNA obtained from EBV-transformed B-cell lines from the C3-deficient patient, her family, and a normal subject. Amplification of cDNA was then carried out by PCR by adding 2.5 units of *Taq* polymerase (Perkin–Elmer/Cetus), 8 μl of dimethyl sulfoxide, 8 μl of PCR supplement buffer [500 mM Tris·HCl, pH 8.3/167 mM $(NH_4)_2SO_4$/100 mM 2-mercaptoethanol], and 100 ng of each primer (oligonucleotide EX20, 5′-TACCGGCAGAACCAAGAGCTC-3′, corresponding to positions 2620–2640; oligonucleotide EX24, 5′-TGTTCCCCGCAGCCCGAGG-3′, corresponding to positions 3080–3098) (16). The samples were amplified by 30 cycles of heat denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. The amplified products were separated on 2% agarose gels, electroeluted, precipitated with

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Abbreviation: EBV, Epstein–Barr virus.
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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M77847, M77848, and M77849).

ethanol, cloned into the *Sma* I site of M13mp18, and sequenced by the dideoxynucleotide chain-termination method (19) with Sequenase (United States Biochemical). The same method was applied to amplify and sequence the β chain of C3 from the cDNA by using two oligonucleotides: oligonucleotide EX3, 5'-ATCCCAGCCAACAGGGAG-3', at nucleotide positions 328–345; and oligonucleotide EX7, 5'-TTATAGATGTAGTAGAATTTC-3', at nucleotide positions 780–800. Part of the C3d region was amplified by using oligonucleotide EX28, 5'-ATGCTCTGGCCCAGATGG-3', corresponding to nucleotides 3641–3658, and oligonucleotide EX30, 5'-TCCTGGTGGTCAGGGGCG-3', corresponding to nucleotides 3915–3932 (16).

PCR Amplification of Genomic DNA and Sequencing. Genomic DNA (1 μg) of the patient and a normal subject was amplified, using as primers the following two oligonucleotides: oligonucleotide EX21B, 5'-AGGGTACCTTCATCAGTGACGGTGTC-3', which contained 18 nucleotides (positions 2818–2835) of exon 21 sequence and an additional *Kpn* I site; and oligonucleotide EX24C, 5'-AGAAGCTTGAGGTGCTTCAGCCGTTC-3', constructed complementary to 18 nucleotides of exon 24 (positions 3052–3069) with an additional *Hin*dIII site. The amplifications were carried out in 50 μl reaction mixtures containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM each dNTP, 0.01% gelatin, 100 ng of each primer, and 2.5 units of *Taq* polymerase. The reactions were run for 35 cycles, each consisting of denaturation at 94°C for 1.5 min, annealing at 50°C for 1.5 min, and extension at 72°C for 3 min. Restriction maps of the amplified products were obtained after single and double digests with restriction enzymes. DNA sequencing of the entire region was performed, as described above, after cloning specific DNA fragments into M13mp18 or M13mp19.

RESULTS

Case Report. The patient is a female Caucasoid, Afrikaans-speaking, South African who is now 34 years old. Her case was reported by Alper *et al.* (1) and she was the first subject to be identified with homozygous C3 deficiency. She developed two episodes of meningococcal meningitis at 5 and 6 years of age. From the age of 6–18 years, she was admitted to hospital an average of two or three times each year with episodes of pneumonia. Each of these infections was followed by development of a self-limiting rash variously described as erythema gyratum perstans (1) and Sweet syndrome (20). Skin biopsy has shown a dense dermal and subdermal neutrophilic infiltrate with leukocytoclasis (1). Since 1985, she has been entirely well and has had no further admissions to hospital and no episodes of rash. She has given birth to three normal children, now 14, 10, and 7 years old. The patient's parents were first cousins, as were her paternal grandparents (21).

Abnormal Structure of the *C3* Null Gene. Genomic DNA from the patient and her family was analyzed by Southern blotting. An 800-bp deletion in the *C3* null gene compared to the normal allele was found in the patient and her family after digestion with *Sst* I and *Eco*RI restriction enzymes. Fig. 1*A* shows that the normal 3.2-kilobase (kb) *Sst* I fragment was replaced by a 2.4-kb fragment in the patient, indicating a partial gene deletion of 800 bp. The presence of both the normal 3.2-kb and unique 2.4-kb *Sst* I fragments in the children of the propositus was consistent with heterozygosity for the partially deleted allele. In addition, *Eco*RI-digested DNA from the patient lacked a normal 5.6-kb band, which was replaced by a larger band of 17 kb (Fig. 1*A*), generated by the loss of an *Eco*RI restriction site from the coding region of exon 23 (11). These findings allowed us to map the 800-bp deletion to a region of the α chain spanning exon 20 (first *Sst* I site at nucleotide 2639) to exon 24 (second *Sst* I site at nucleotide 3203) (16) (Fig. 1*B*). We then used two approaches, one at the mRNA level and one at the genomic level, in order to define the detailed structure of the deletion.

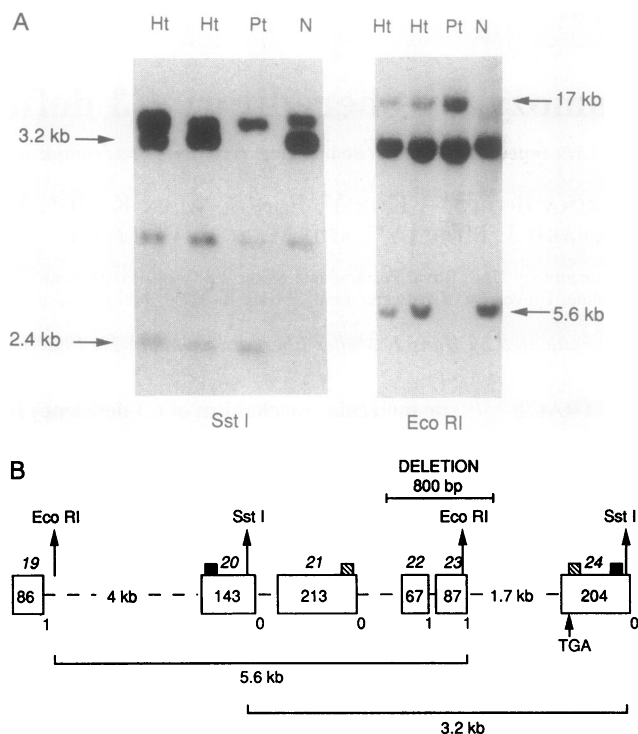

FIG. 1. (*A*) Southern blot analysis of peripheral blood genomic DNA from the patient (lanes Pt), two of the heterozygous children (lanes Ht), and a normal subject (lanes N). DNA (10 μg) was completely digested with *Eco*RI and *Sst* I restriction enzymes and probed with pC3.11. *Sst* I-digested DNA showed the absence of the normal 3.2-kb fragment and the presence of a unique 2.4-kb band in the patient (lanes Pt). The heterozygotes had both the fragments. Southern hybridization analysis of *Eco*RI-digested DNA revealed the loss of an *Eco*RI restriction site in the mutant allele and replacement of the normal 5.6-kb fragment by a larger fragment of 17 kb. Fragment sizes are indicated. (*B*) Schematic representation of the *C3* gene showing the location of the deletion. Exons 19–24 are indicated by open boxes and the numbers within refer to their length in nucleotides. The numbers at the bottom right corner below each box refer to intron type: type 0 introns interrupt between codons and type 1 interrupt after the first nucleotide. Solid and hatched squares represent positions of the oligonucleotide primers used in PCR amplification of the *C3* from cDNA and genomic DNA, respectively. Restriction sites for *Eco*RI and *Sst* I are shown by vertical arrows and the sizes of the digested fragments are indicated by horizontal lines at the bottom of the figure.

Sequence Analysis of the Abnormal *C3* cDNA. First-strand cDNA synthesis was performed with mRNA extracted from EBV-transformed cell lines as a template. Segments of this were amplified by PCR using target sequences encoding different regions of the C3 molecule. Amplification of the region spanning exons 20–24 (using oligonucleotides EX20 and EX24 as primers) from the patient generated a product that was 154 bp smaller than that found in the normal subject (Fig. 2). Both the normal and the short PCR products were detected from the cDNA of the patient's children (data not shown), who were heterozygous for the deletion (Fig. 1*A*). Amplification of two other regions of the cDNA 5' and 3' to the deletion (spanning exons 3–7 in the β chain and exons 28–30 in the α chain) generated products of identical size from both the patient and a normal subject (Fig. 2).

The abnormal cDNA fragment from the patient was sequenced and a 154-bp deletion was found (from nucleotides 2857–3110). The deleted fragment included two entire coding regions (exons 22 and 23). Because introns 21 and 23 differ in

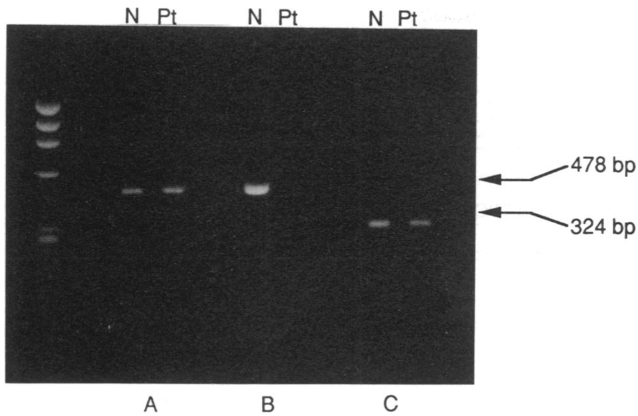

FIG. 2. Agarose gel electrophoresis of PCR products. Amplified products from *C3* cDNA derived from lipopolysaccharide-stimulated EBV-transformed cell lines were run on a 2% agarose gel. Paired lanes A, B, and C represent regions spanning exons 3–7 in the β chain, exons 20–24 over the deletion, and exons 28–30 in the C3d region, respectively. Lanes N and Pt, samples from a normal subject and from the patient, respectively. *Hae* III-digested bacteriophage ϕX174 were run as size standards. Paired lanes B showed bands of 478 (lane N) and 324 (lane Pt) bp, revealing a difference of 154 bp in the coding region. Both PCR products in lanes B were subsequently sequenced.

their phases [respectively, type 0 (interrupting two exons between codons) and type 1 (interrupting two exons after the first nucleotide of a codon)] (Fig. 1*B*), the resulting mRNA was out of the normal translation frame distal to the deletion generating a stop codon (TGA) 19 bp downstream of the 5′ end of exon 24 (Fig. 3).

Junctions of the Genomic Deletion. Genomic DNA spanning exons 21–24 from the patient and a normal subject were amplified by PCR in order to localize precisely the breakpoints of the deletion. PCR products of 2.8 and 2 kb, respectively, were generated from normal and from patient DNA (Fig. 4). Amplified fragments were mapped by single and double digests and sequenced, after subcloning into M13mp18 and M13mp19. The sequence of normal genomic DNA showed the presence of two *Alu* sequences, one located in intron 21 and one in intron 23. These were oriented in the opposite direction with respect to the *C3* gene.

The sequence of the mutant allele showed the deletion of exons 22 and 23, and the structure of the intron between exons 21 and 24 was a hybrid between the normal intron 21 and 23 sequence. The sequence of this intron changed from that of normal intron 21 to that of normal intron 23 within the two *Alu* sequences (Fig. 5). Comparison of the sequences suggests that the mutant sequence may have arisen by homologous recombination between the two *Alu* sequences within a region of 19 nucleotides, located in the left arm of the *Alu* repeat between the A and B promoter sites of RNA polymerase III. Therefore, the deletion event has resulted in a reconstituted *Alu* sequence (Fig. 5) (22).

Frequency of the *C3* Null Gene in the Afrikaner Population of South Africa. It was noted, as part of the original family study of the propositi, that there was a nonconsanguineous relative of the patient who had heterozygous deficiency of *C3* (21). We obtained a blood sample from this individual and showed that she was also heterozygous for the identical *C3* gene deletion. We then went on to ascertain the prevalence of the deleted allele in a consecutive series of 174 Afrikaans-speaking white South Africans attending a casualty department (with informed consent and permission of the local research ethics committee). Southern blot analysis was performed using the *Sst* I restriction enzyme. The same gene deletion was identified in two of these individuals, giving a gene frequency of 0.0057. These individuals expressed *C3* levels of 66% and 69% of a normal human serum pool and were allotyped as *C3S*.

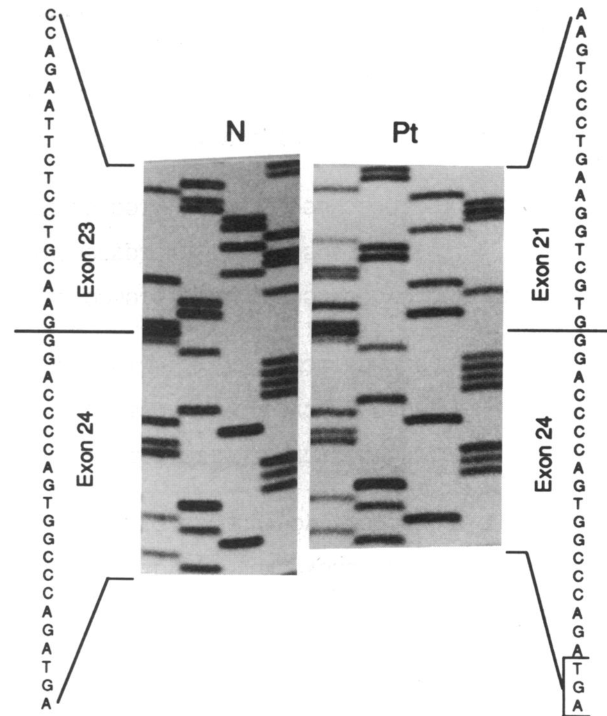

FIG. 3. Autoradiograph of sequence from exons 20–24. Amplified DNA obtained from PCR of *C3* cDNA was sequenced as described in the text. Amplified cDNA from the patient showed a 154-bp deletion resulting from deletion of exons 22 and 23. On the left is shown the normal (N) cDNA sequence running from exon 23 to exon 24. On the right is the sequence from the patient (PA) showing the junction between exons 21 and 24. A frameshift results from the splicing of exon 21 to exon 24, due to the differing phases of introns 21 and 23 [respectively, types 0 (interrupting between codons) and 1 (interrupting after the first nucleotide of a codon)]. As a result of the frameshift a stop codon, TGA (bracketed), is generated 19 bases downstream from the 5′ end of exon 24.

DISCUSSION

In this paper, we describe the molecular basis of homozygous *C3* deficiency in an Afrikaans patient. There was an 800-bp

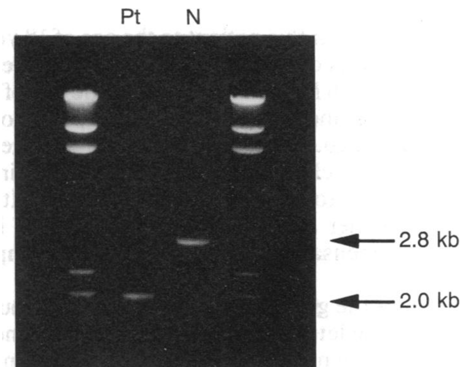

FIG. 4. Agarose gel electrophoresis of PCR-amplified genomic DNA. Genomic DNA from the patient (lane Pt) was amplified by PCR. Amplified products, spanning exons 21–24, were run on a 2% agarose gel. *Hin*dIII-digested bacteriophage λ were used as size standards. The PCR product obtained from the patient's DNA was 2 kb, showing a deletion of 800 bp compared with the fragment found in the normal subject (lane N) (2.8 kb). Both PCR products were sequenced.

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                                                   ↓
Intron 21   ATTTATTTATTTATTTATTTATTTATTTATTTTTTTGAGACAGAGTCTCACTC
                                *   *
Mutant      ATTTATTTATTTATTTATTTGTTTGTTTATTTTTTGAGACAGAGTCTCACTC
            * * *  *** **    ** *   *   *                **
Intron 23   CTGTTTTCCCTCTTTTACATTTTTTTTTTTTTTTGAGACAGAGTCTGGCTC

Intron 21   TGTTCCCCAGGCTGGAGTGCAGTGGCGCGATCTCAGCTCACTGCAATATCCGC

Mutant      TGTTCCCCAGGCTGGAGTGCAGTGGCGCGATCTCAGCTCACTGCAATATCCGC
               **                       *     * *      **
Intron 23   TGTCACCCAGGCTGGAGTGCAGTGGCGCGACCTCAGCCCGCTGCAAGCTCCGC

Intron 21   CTCCCGGGTTCACGCCATCCTCCTGCCTCAGCCTCCCGAGTAGCTGGGACTAC

Mutant      CTCCCGGGTTCACGCCATCCTCCTGCCTCAGCCTCCCGAGTAGCTGGGACTAC
                              *
Intron 23   CTCCCGGGTTCACGCCATTCTCCTGCCTCAGCCTCCCGAGTAGCTGGGACTAC

Intron 21   AGGCGCCCCCACCACGCCCGGCTAATTTTTTTGTATTTTTAGTAGAGACAGGG

Mutant      AGGCGCCCCCACCACGCCCGGCTAATTTTTTTGTATTTTTAGTAGAGACAGGG
              ***
Intron 23   AGCACCCGCCACCACGCCCGGCTAATTTTTTTGTATTTTTAGTAGAGACAGGG

                                      B
Intron 21   TTTCACTGTGTTAGCCAGGATGGTCTCGATCTCCTGACCTCGTGATCCACCCA
                                                                *
Mutant      TTTCACTGTGTTAGCCAGGATGGTCTCGATCTCCTGACCTCGTGATCCACCCG
                  *                        *
Intron 23   TTTCACCGTGTTAGCCAGGATGGTCTCGATCTTCTGACCTCGTGATCCACCCG

                                                    A         ↓
Intron 21   CCTCGGCCTCCCAAAGTGCTAGGATCACAGGCATGAGCCACCGCGCCCGGCC-
                *              *   *             ******      *
Mutant      CCTCAGCCTCCCAAAGTGCTGGGATTACAGGCATGAGCATTAT-GCCCGGCCT

Intron 23   CCTCAGCCTCCCAAAGTGCTGGGATTACAGGCATGAGCATTAT-GCCCGGCCT

Intron 21   AATGCTAGGGTGATCCCTAAGGACAGTGCCCTGCTGACCATCTGTGTGTCTGT
            *** ****  *  *   *   **   *  *  ****        ****    *    *  ****  *
Mutant      AAAAATTTTTTTAACCATACAGATATTATT-TGCTATTGATCGGTTTTATAGA

Intron 23   AAAAATTTTTTTAACCATACAGATATTATT-TGCTATTGATCGGTTTTATAGA
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FIG. 5. DNA sequence across deletion junctions in the *C3* mutant allele and the corresponding regions of the normal gene. The normal sequence of intron 21 (top line), the sequence across the deletion junctions (middle line), and the normal sequence of intron 24 (bottom line) are aligned and gaps (−) were inserted to achieve maximum homology. Asterisks between sequences denote positions at which the base pairs differ. A and B consensus sequences for RNA polymerase III promoters are overlined. The presumed recombination site is enclosed in a box. Vertical arrows delineate the *Alu* sequence repeat as defined by the consensus sequence (22). Note that the *Alu* sequences are in the opposite orientation to the *C3* gene.

deletion in the gene, including the whole of exons 22 and 23 of the gene. Analysis of cDNA from a B-lymphoblastoid cell line derived from the patient demonstrated that C3 mRNA amplified by PCR was truncated and out of frame as a consequence of loss of these two exons in the α chain. This mutation is not compatible with the production of any functional C3 protein.

The clinical history of this patient to the age of 18 years has been reported (1). It is of note that the frequency of episodes of pneumonia and rash has decreased since the age of 18. She is now 34 years old and her last episode of pneumonia and rash was 6 years ago. There has been no change in her environment and the clinical consequences of her immunodeficiency state seem to be diminishing as she ages. It may be hypothesized that her adaptive immune system is increasingly able to compensate for defective innate complement activity.

Investigation of the genomic structure of the *C3* null allele showed that the deletion probably arose by homologous recombination between two *Alu* sequences located in introns 21 and 23, resulting in a null allele with two deleted exons and a hybrid *Alu* sequence. The recombination probably occurred within a region of identity of 19 nucleotides in the intron 21 and 23 *Alu* sequences. This region is located in the left arm of the *Alu* repeat in an area implicated in transcription by RNA polymerase III (23).

Alu repeats have been associated with recombination events, homologous or nonhomologous, that caused deletions or duplications in a large number of human genes including low density lipoprotein receptors (23); γ-, δ-, and β-globin (24, 25); apolipoprotein B (26); α_2-plasmin inhibitor (27); C1 inhibitor (28); adenosine deaminase (29); α-galactosidase (30); lysosomal β-hexosaminidase (31); and others. Lehrman and colleagues (23) noted that the majority of these recombination events involved the left half of the *Alu* repeat sequence, particularly the region between the A and B sequences. These sequences are homologous to the two halves of the RNA polymerase III split promoter. It has been shown, by using *in vitro* assays, that many *Alu* elements are active in RNA polymerase III transcription assays (32); it is not known whether this is also true *in vivo*. It is likely that these promoter-like sequences play a role in recombination, since the majority of recombination events involving *Alu* repeats have been described as occurring between the A and B sequences, including the deletion reported in this paper.

In the original family study of the propositos a nonconsanguineous relative was identified who had heterozygous *C3* deficiency (21) and Alper and colleagues speculated that the prevalence of the null allele for *C3* may be quite high among the Afrikaans population. To address this question, we studied a group of 174 consecutive Afrikaans-speaking Caucasoid subjects attending a casualty department in South Africa. Among this small population, we identified an additional two unrelated individuals who were heterozygous for the deletion. This gives a gene frequency for the *C3* null allele of 0.0057 and from this it may be calculated that there should

be ≈81 individuals with homozygous *C3* deficiency among a population of ≈2.5×10^6 (33). Where are these individuals? Another patient with *C3* deficiency has been reported from Pretoria (2), a young Caucasoid girl with recurrent pneumococcal meningitis and pneumonia, who died at the age of 7 years of pneumococcal meningitis. A diagnosis of *C3* deficiency should be considered, particularly among Afrikaans patients with recurrent pyogenic infections, especially when accompanied by atypical rash. The alternative possibility is that there may have been positive selection for subjects with heterozygous *C3* deficiency among casualty attenders. Many heterozygotes have been identified among the families of patients with homozygous *C3* deficiency and there is no published evidence that they have increased susceptibility to infectious disease, although there is a single report of a Lebanese family in which two heterozygotes suffered from nephritis (6).

The Afrikaners are a distinctive population of white South Africans whose ancestry has been traced to a small group of Dutch, German, and Huguenot settlers who founded the Cape Colony in the late 17th and early 18th centuries (33). They form a relatively inbred community as a result of past breeding patterns and have an unusually high prevalence of certain rare inherited diseases. These include familial hypercholesterolemia, porphyria variegata, progressive familial heart block, sclerosteosis, lipoid proteinosis, and Huntington chorea (33, 34). A combination of the founder effect, intermarriage, and genetic drift is likely to be the explanation for this high prevalence of certain inherited diseases. The present findings add another member to the unique pattern of genetic disorders prevalent among the Afrikaans population of South Africa. *C3* deficiency has also been described in a Dutch family (8), and we wondered if this might be due to the same mutation. However, our initial investigations on a *C3* deficiency member of this Dutch family did not show the same deletion found in the South African patient (data not shown).

In conclusion, we have identified a small genomic deletion as the cause of homozygous *C3* deficiency in an Afrikaans patient. This probably arose because of homologous recombination in which unequal crossing-over resulted from mispairing of homologous *Alu* sequences during meiosis. This abnormal gene has achieved a relatively high frequency in the Afrikaner population of South Africa, probably through the founder effect mechanism.

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