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Linking C5 Deficiency to an Exonic Splicing Enhancer Mutation

Nicole Pfarr,1* Dirk Prawitt,1* Michael Kirschfink,1† Claudia Schroff,* Markus Knuf,* Pirmin Habermehl,* Wilma Mannhardt,* Fred Zepp,* William Fairbrother,† Michael Loos,§ Christopher B. Burge,‡ and Joachim Pohlenz2*

As an important component of the innate immune system, complement provides the initial response to prevent infections by pathogenic microorganisms. Patients with dysfunction of C5 display a propensity for severe recurrent infections. In this study, we present a patient with C5 deficiency demonstrated by immunochemical and functional analyses. Direct sequencing of all C5 exons displayed no mutation of obvious functional significance, except for an A to G transition in exon 1 predicting an exchange from lysine to arginine. This sequence alteration was present in only one allele of family members with a reduced serum C5 concentration and in both alleles of the patient with almost complete C5 deficiency, suggesting that this alteration may be producing the phenotype. Recent findings indicate that distinct nucleotide sequences, termed exonic splicing enhancers (ESEs), influence the splicing process. cDNA from all family members harboring the mutated allele showed skipping of exon 10, which resulted in a premature STOP codon, explaining the lack of C5 in the propositus. Sequence analysis of the mutated region revealed the substitution to be located within an ESE, as predicted by the RESCUE-ESE program. The altered ESE sequence is located close to the 5’ splicing site and also lowers the predicted strength of the splice site itself. This apparently inconsequential sequence alteration represents a noncanonical splicing mutation altering an ESE. Our finding sheds a new light on the role of putative silent/conservative mutations in disease-associated genes. The Journal of Immunology, 2005, 174: 4172–4177.

Complement plays a vital role in the body’s immune system, providing a highly effective means for the destruction of invading microorganisms and for immune complex elimination (for review, see Refs. 1 and 2). Complement may be activated via the classical, the alternative, or the lectin (i.e., mannose-binding lectin and ficolins) pathways. All lead to cleavage of C3, into C3a and C3b. Binding of C3b enables a better clearance of pathogens and immune complexes as well as the generation of the lytic membrane attack complex, C5b-9. C5, the fifth component of the complement system is a 190,000 Mr glycoprotein consisting of 1666 aa in two disulfide-linked polypeptide chains, C5α and C5β. After activation by the C5 convertases, C5 is cleaved into C5α and C5β. C5α is the most potent complement-derived proinflammatory peptide (3, 4). It serves not only as a chemoattractant but also initiates multiple defense mechanisms in leukocytes. Through its multiple binding sites C5α initiates and directs the assembly of the membrane attack complex.

C5 is encoded by a single gene copy on chromosome 9q34.1 which contains 41 exons that span a genomic region of 79 kb. The gene encodes the open reading frames for C5α (exon 1–16), as well as for C5β (exons 16–41). Both are transcribed as a single 6.0 kb mRNA and translated into a pre-C5 protein (in β-α orientation). These single chain precursors are processed into the mature form of C5 by the removal of the four internal residues to yield a two-chain protein (5).

Through activation and interaction with respective receptors on various immune cells, complement is closely linked to the adaptive immune response. A normally functioning complement system is also required for physiological tissue regeneration and repair. Complement defects increase the susceptibility to infection and are frequently associated with autoimmune disorders (6, 7). Patients with dysfunction of C5 display a propensity for severe recurrent infections, particularly with Neisseriae, the causative pathogen for meningitis and extragenital gonorrhea. Furthermore, C5 deficiency produces an anti-inflammatory phenotype that may also be involved in certain forms of asthma (8).

Hereditary C5 deficiency has been reported in several families of different ethnic backgrounds and from different geographic regions (7). To date, only two heterozygous nonsense mutations of the human C5 (hC5)3 gene have been reported to account for complete C5 deficiency. Whereas in two C5-deficient families of African-American origin a nonsense mutation located in exon 1 (Gln1 to STOP) was found, another mutation in exon 36 (Arg1458 to STOP) was identified in a third family. Because the affected individuals reported so far were heterozygous for the mutations and thus are believed to be able to express one normal C5 allele, it was speculated that a complete C5 deficiency requires additional molecular defects (9). The majority of recognized mutations which cause diseases occur in the coding sequences of genes with a direct

Abbreviations used in this paper: hC5, human C5; ESE, exonic splicing enhancer; CH50, hemolytic activity of the classical pathway; AH50, hemolytic activity of the alternative pathway; PMN, polymorphonuclear neutrophil; SNP, single nucleotide polymorphism; GH1, growth hormone type 1; CF, cystic fibrosis.

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2 Address correspondence and reprint requests to Dr. Joachim Pohlenz, Children’s Hospital of Johannes Gutenberg-University of Mainz, Langenbeckstrasse 1, Building 109, D-55101 Mainz, Germany. E-mail address: pohlenz@kinder.klinik.uni-mainz.de
3 Abbreviations used in this paper: hC5, human C5; ESE, exonic splicing enhancer; CH50, hemolytic activity of the classical pathway; AH50, hemolytic activity of the alternative pathway; PMN, polymorphonuclear neutrophil; SNP, single nucleotide polymorphism; GH1, growth hormone type 1; CF, cystic fibrosis.
impact on the encoded protein structures and their function. However, another probably large and yet unrecognized proportion of diseases are caused by genetic aberrations resulting from mutations that influence gene transcription, including mRNA processing, export, stability, as well as translational control (10). Based on the fact that alternatively spliced variants have a tissue-specific distribution, alternative splicing has been also considered as a reason for compound heterozygous C5 deficiency with only one open-reading frame being mutated. So far, none of the analyzed splice sites responsible for pre-mRNA processing harbored a mutation that could lead to incorrect splicing of the hC5 gene. However, recent findings indicate that exonic sequences not only encode for protein information, but also contain sequences essential for correct mRNA splicing. These specific short oligonucleotide sequences, termed exonic splicing enhancers (ESEs), increase pre-mRNA splicing and, therefore, play an important role in constitutive and alternative splicing. Such ESEs often activate specific splice sites to facilitate the recognition of an exon by the splicing apparatus and thus, result in exon skipping. Therefore, gene sequence analysis has to be extended to enable the determination of the impact of a sequence alteration on putative ESE motifs.

In this study, we report a new case with selective near total C5 deficiency as revealed by immunochemical and functional analyses. Sequencing of the hC5 coding region did not explain the lack of C5 protein because the mutation, a conservative amino acid exchange from an arginine to a lysine codon, was not expected to produce a functional defect. However, the identified sequence alteration that affects splicing signals (either by ESE alteration or by creation of a new splice acceptor) that could lead to incorrect splicing of the hC5 gene. However, recent findings indicate that exonic sequences not only encode for protein information, but also contain sequences essential for correct mRNA splicing. These specific short oligonucleotide sequences, termed exonic splicing enhancers (ESEs), increase premRNA splicing and, therefore, play an important role in constitutive and alternative splicing. Such ESEs often activate specific splice sites to facilitate the recognition of an exon by the splicing apparatus and thus, result in exon skipping. Therefore, gene sequence analysis has to be extended to enable the determination of the impact of a sequence alteration on putative ESE motifs.

In this study, we report a new case with selective near total C5 deficiency as revealed by immunochemical and functional analyses. Sequencing of the hC5 coding region did not explain the lack of C5 protein because the mutation, a conservative amino acid exchange from an arginine to a lysine codon, was not expected to produce a functional defect. However, the identified sequence alteration that affects splicing signals (either by ESE alteration or possibly by changing the 5' splicing site itself), leading to exon skipping and a truncated transcript, is likely responsible for the observed phenotype.

Materials and Methods

Patient

The propositus, a 5-year-old boy, is the third child of consanguineous Turkish parents. In the first year of life, he underwent cardiac surgery because of coarctation of the aorta and a ventricular septal defect. His development proceeded normally until the age of 3 years when he was admitted to the hospital because of headache, fever, vomiting, and abdominal purpura. Meningitis was suspected but cultures of the cerebrospinal fluid were negative. *Neisseria meningitides* was isolated in blood cultures (Serva), and adjusted to 1 x 10^6 cells/ml. Viability of PMN was found to be >98% as judged from trypan blue staining. A total of 0.9 ml of the cell suspensions (1 x 10^3/ml) was added to the upper compartment of the chemotaxis chamber separated by a nitrocellulose membrane (3.0 μm pore size, 13 mm in diameter, 140 μm thickness; Sartorius) from the lower compartment, containing 0.2 ml of the chemoattractant or buffer as control. All samples and cell suspensions were frozen at -70°C until assayed. After complement C5 deficiency was diagnosed, the patient was placed on long-term prophylaxis with oral penicillin. Since then, he had no further recurrence of severe infections. His parents and a brother had decreased serum levels of complement C5 (Table 1), but no history of severe infections. All family members gave written informed consent to undergo molecular studies.

Table I. Complement profile of the patient and his family

<table>
<thead>
<tr>
<th>Test</th>
<th>Normal Range</th>
<th>Patient</th>
<th>Mother</th>
<th>Father</th>
<th>Sister</th>
<th>Brother</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH50 (%)</td>
<td>80–120</td>
<td>&lt;1</td>
<td>50</td>
<td>79</td>
<td>81</td>
<td>91</td>
</tr>
<tr>
<td>AH50 (%)</td>
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<td>80</td>
<td>84</td>
<td>92</td>
</tr>
<tr>
<td>C5 function (%)</td>
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<td>42</td>
<td>48</td>
<td>95</td>
<td>45</td>
</tr>
<tr>
<td>C5 (μg/ml)</td>
<td>70–160</td>
<td>&lt;0.05</td>
<td>83</td>
<td>73</td>
<td>127</td>
<td>85</td>
</tr>
<tr>
<td>C6–C9 function (%)</td>
<td>100</td>
<td>200</td>
<td>170</td>
<td>119</td>
<td>168</td>
<td>146</td>
</tr>
<tr>
<td>SC5b-9 (ng/ml)</td>
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<td>&lt;1</td>
<td>20</td>
<td>30</td>
<td>67</td>
<td>41</td>
</tr>
<tr>
<td>C3 (mg/ml)</td>
<td>0.75–1.4</td>
<td>1.24</td>
<td>0.95</td>
<td>1.23</td>
<td>0.84</td>
<td>1.22</td>
</tr>
<tr>
<td>C3d (mU/ml)</td>
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<td>10</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>C4 (mg/ml)</td>
<td>0.1–0.34</td>
<td>0.16</td>
<td>0.12</td>
<td>0.37</td>
<td>0.12</td>
<td>0.27</td>
</tr>
</tbody>
</table>

* Functional and immunochemical analyses indicate an almost complete C5 deficiency in the patient.

Pooled normal serum served as standard (defined as 100%).

Immunological analyses

Hemolytic assays. Functional activity of the classical (CH50, Ref. 11) and the alternative pathway (AH50, Ref. 12) of complement was measured according to described procedures. Hemolytic activity of C5 was tested using C5-deficient human serum (Calbiochem Merck Biosciences). C6-C9 function was titrated with Ab-sensitized sheep erythrocytes (EA), preincubated with C6-deficient rabbit serum to collect EAC1–5. Plasma concentrations of C4 and C3 were determined by nephelometry (Behring). C3d/C3d was measured by double-decker rocket immunoelctrophoresis using rabbit anti-C3c in the lower and rabbit anti-C3d Abs (DAKO) in the upper gel (13). C5 protein concentration was determined by ELISA: in brief, microtiter plates (Nunc) were coated with rabbit anti-human C5 IgG (DAKO). After blocking nonspecific binding sites with 1% BSA in PBS, appropriate dilutions of the samples were added. Following incubation, C5 was detected by a monoclonal mouse anti-human C5 IgG (Quidel), followed by the appropriate peroxidase-labeled third Ab (Dianova). The reaction was visualized by the addition of o-phenylenediamine/H2O2 substrate. Purified C5 (Calbiochem) was used as standard. SC5b-9 ELISA was performed as described before (14). In some experiments, the patient’s and control serum samples were preactivated with zymosan to generate SC5b-9 as a measure of terminal activation pathway.

C5 reconstitution. C5 reconstitution assays were performed to investigate restoration of total hemolytic activity (CH50) and chemotactic activity by addition of incremental amounts of purified C5 (Sigma-Aldrich) to the patient’s serum.

SDS-PAGE and Western blot analysis. To further investigate the presence and protein structure of the patient’s C5, serum (native and immunoprecipitated with rabbit anti-C5 IgG; DAKO) was subjected to SDS-PAGE (15) under nondenaturing and denaturing conditions. Proteins were transferred onto a nylon membrane (16) and probed by chemiluminescence-enhanced Western blot and immunodetection of C5-specific bands with various monoclonal and polyclonal C5-specific Abs.

Chemotaxis assay. Chemotaxis of polymorphonuclear neutrophil (PMN) was evaluated by using a two-compartment, blind-well Boyden chamber (17). A total of 20 ml of heparinized venous blood from healthy donors was layered on a sodium metrizoate/dextran gradient (Polyomorphoprep; Immun) in HBSS. After centrifugation at 470 x g for 30 min, the PMN-rich cell layer was washed with HBSS (without Ca2+ and Mg2+) and residual erythrocytes were removed by hypotonic lysis. Subsequently, the cell pellets (>98% purity) were resuspended in HBSS, containing 0.25% BSA (Serva), and adjusted to 1 x 10^6 cells/ml. Viability of PMN was found to be >98% as judged from trypan blue staining. A total of 0.9 ml of the cell suspensions (1 x 10^3/ml) was added to the upper compartment of the chemotaxis chamber separated by a nitrocellulose membrane (3.0 μm pore size, 13 mm in diameter, 140 μm thickness; Sartorius) from the lower compartment, containing 0.2 ml of the chemoattractant or buffer as control. All samples and cell suspensions were frozen at -70°C until assayed. After complement C5 deficiency was diagnosed, the patient was placed on long-term prophylaxis with oral penicillin. Since then, he had no further recurrence of severe infections. His parents and a brother had decreased serum levels of complement C5 (Table 1), but no history of severe infections. All family members gave written informed consent to undergo molecular studies.

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<td>16</td>
</tr>
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<td>0.27</td>
</tr>
</tbody>
</table>

* Functional and immunochemical analyses indicate an almost complete C5 deficiency in the patient.

Pooled normal serum served as standard (defined as 100%).
in HBSS with 2 mM Ca\(^{2+}\)/0.8 mM Mg\(^{2+}\) (HBSS\(^{2+}\)) and 0.25% BSA, pH 7.35. To analyze restoration of chemotactic activity, one aliquot of the patient’s serum was resubstituted with C5 to receive a final concentration corresponding to that in normal human serum (75 µg/ml). After incubation for 75 min at 37°C, filters were removed, fixed in 100% propanol, stained with hematoxylin, and mounted on microscope slides.

Migration into the filter was quantified by the leading front method (18). Duplicate chambers were used in each experiment and four microscopic fields (×400) for each filter were semiautomatically evaluated by an image analyzer (VRZ-U2; Leitz). Buffer control (random cell movement) was subtracted from each sample, and data are presented as migration distance (µm/75 min).

Genetic analyses
Preparation of RNA and DNA amplification and sequencing the hC5 gene. Genomic DNA from all available family members was isolated from peripheral blood using the QIAamp DNA Blood Mini kit (Qiagen). Coding sequences of the hC5 gene were then amplified with primers flanking all 41 exons and the promoter region 5' of exon 1 (PCR conditions and primer sequences will be supplied upon request). The PCR products were purified by isopropanol precipitation and sequenced using an automated sequencing system (A377; Applied Biosystems). RNA was prepared from lymphocytes and isolated using the QIAamp RNA Blood Mini kit (Qiagen), avian myeloblastosis virus reverse transcriptase (Promega), and a mixture of oligo(dT) primers and random hexamers were used to synthesize the first strand of cDNA from lymphocyte-derived total RNA, according to the manufacturer’s protocols. Oligonucleotide primers for amplification and sequencing the part of the hC5 DNA encompassing exons 9–12 were C5-9f(5'-CAGCAGTCAAAGAACTGTCA-3') and C5-12r(5'-TCAGATGTCTTCCACTAGC-3'). With these primers, amplification of cDNA containing the wild-type allele is expected to result in a 540-bp fragment.

Sequence analysis. Obtained sequences containing the exonic sequences, intron/exon boundaries, and putative promoter sites were compared with the published wild-type sequence (Accession no. NM_001735) using the SEQUENCER (Gene Codes) computer program that allows detection of mutations/polymorphisms. Variations were then annotated with predicted ESEs using the RESCUE-ESE web server (http://genes.mit.edu/burgelab/rescue-ese/). The RESCUE method relies upon the assumption that sequences that enhance the recognition of a splice site will occur more frequently in the vicinity of suboptimal splice sites than consensus splice sites. Although the details of the RESCUE protocol have been described previously (19), the basic approach was to count hexamer frequencies in databases of exons and introns that have been sorted according to splice site strength. The splice site scores were used to separate the exons database into “weak” and “strong” sets. The weak exon dataset was composed of the exons that were adjacent to splice sites that score in the bottom quartile of all the splice sites tested while the strong exons had splice sites that scored in the top quartile. As each exon is flanked by a 3’ splice site and a 5’ splice site, there were a total of four new sets created from the original exon database. The t statistic was used to identify cases of exons that had significantly different counts in: 1) exons relative to introns and 2) exons with weak (low scoring) splice sites relative to exons with strong (high scoring) splice sites. A list of hexamers and t scores was received which enabled the identification of the subset of exons that were significantly enriched in sequences that were both exonic and adjacent to a weak splice site (20).

Wild-type and mutant sequences of C5 were scored for agreement to the splice site consensus using a maximum entropy model for human splice sites (20).

Results
Functional activity of the component pathway
Functional activity of CH50 and AH50 in the patient’s serum hemolytic activity was almost undetectable (Table I), suggesting a deficiency of one of the terminal complement components, C5-C9. Functional and immunochemical assays revealed a deficiency of the fifth component of the complement system (Table I), while C3, C4, and C6-C9 were normal. Normal plasma levels of the C3 split product C3d/C3d excluded a secondary deficiency due to complement consumption. In vitro C5 reconstitution fully restored total hemolytic activity (CH50, Fig. 1).

Complement C5 deficiency
C5 ELISA revealed only trace amounts of C5 in the patient’s serum (Table I), providing the basis for close to zero C5 and CH50 activities. Therefore, we were not able to further characterize C5 protein structure by SDS-PAGE despite application of sensitive chemoluminescence-enhanced Western blot analysis. Zymosan activation of the patient’s serum led to a generation of only 22 ng/ml SC5b-9 in comparison to ~120 µg/ml obtained in normal human control serum, further supporting the presence of only trace amounts of functionally active C5 in the patient’s serum.

As expected, only negligible chemotactic activity could be generated in the patient’s serum upon yeast activation. The addition of purified C5 restored (C5a-mediated) chemotactic activity to a level observed in normal serum (Fig. 2).

Mutations in the hC5 gene are associated with C5 activity
Direct sequencing of the hC5 gene of the propositus which included all exons and flanking regions including the promoter region revealed only a substitution of the normal adenine 1115 with a guanine, according to the sequence of Haviland et al. (5) (NM_001735). This mutation predicts the replacement of a normal lysine (K) with an arginine (R) in codon 372 (K372R), both belonging to the group of alkaline amino acids. Whereas the propositus was homozygous for the mutation, both parents and an older brother were heterozygous carriers of K372R. A sister was homozygous for the wild type. The mutation could not be detected in fifty normal individuals from the same ethnic background.

C5 mutation affects a putative ESE
We used the RESCUE-ESE program (19) to evaluate whether the sequence alteration affects an ESE and to determine its possible impact on the quality of the ESE. Indeed, the nucleotide exchange was likely to affect but not to abolish an ESE (ATCAAG into CATCAG, underlining indicates the position of the exchanged nucleotide in respect of its position in the hexamer sequence of the

FIGURE 1. In vitro C5 reconstitution fully restored total hemolytic activity (CH50) of the patient’s serum.

FIGURE 2. Chemotactic activity in the patient’s serum upon yeast activation compared with two control sera. The patient’s sample exerted only negligible chemotactic activity. Addition of purified C5 before activation restored chemotactic activity to a level as observed in normal serum.
FIGURE 3. Statistical analysis of wild-type and mutant sequences of the hC5 gene. A, Wild-type and mutant sequences were separated into two sets of six overlapping hexamers. B, For each hexamer, enrichment was calculated by comparing hexamer frequencies in exons to those in introns (exons vs introns (x-axis); weak exons flanked by weak sites relative to exons flanked by strong sites (y-axis)) and calculating a t score (see Table II) which is labeled dEI (dEI is the ratio of hexamer occurrence in weak exons vs hexamer occurrence in introns). A positive value of dEI reflects an exonic enrichment for that particular hexamer whereas a negative value indicates an intronic enrichment. The analogous procedure was used to calculate enrichment in weak (nonconsensus) splice sites relative to strong (consensus) splice sites. Likewise a positive value of dWS indicates enrichment in weak exons (dWS is the ratio of hexamer occurrence in weak exons vs hexamer occurrence in strong exons). Wild-type sequences are represented by blue squares. Mutant sequences are represented by red squares. Hexamers derived from wild-type sequence (blue squares) are distributed more toward the upper right (enriched in both the weak splice site and the exon set) than the mutant sequence (red squares).

TABLE II. T score statistical analysis of wild-type and mutant sequences of the hC5 gene (dEI)*

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</tbody>
</table>

*For each hexamer, enrichment was calculated by subtracting hexamer frequencies in exons from hexamer frequencies in introns. T scores from all four comparisons are listed with the following abbreviations: E = exon, I = intron, W = weak, and S = strong and so (dEI3 = exon vs intron at 3’ splice site, dEI3WS = weak vs strong at 3’ splice site, dEI5 = exon vs intron at 5’ splice site and dEI5WS = weak vs strong at 5’ splice site). These t scores are listed for each hexamer altered by the mutation (mutated site in bold). A positive value of dEI3 would indicate that in the exon vs intron comparison the hexamer is enriched in exons and a negative value would indicate that the hexamer is enriched in introns.

exons, the t statistic was used again to identify hexamers that occurred more frequently in exons that were defined by suboptimal splice sites than exons adjacent to consensus sites. Both these comparisons were arranged such that positive t scores reflect enrichments that are consistent with ESEs (i.e., ESEs are enriched in exons relative to introns; ESEs are enriched in weak exons relative to strong exons). To better understand this ESE alteration we examined the t scores of the set of six overlapping hexamers that were altered by this mutation. In other words, we compared the degree to which each hexamer in the wild-type and mutant allele of the sequence was enriched in the attributes that define ESEs (Fig. 3A). As shown in Fig. 3B, hexamers derived from wild-type sequence (blue squares) are distributed more toward the upper right (enriched in both the weak splice site and the exon set) than those of the mutant sequence (red squares). This difference is consistent with the idea that the wild-type sequence is more likely to possess ESE activity than the mutant sequence.

C5 mutation alters mRNA splicing

We then isolated total RNA from lymphocytes of all family members and amplified the region from exons 9 to 12 as cDNA. Indeed, in all individuals having at least one normal allele we detected the expected normal 540 bp band. However, the propositus did not have the expected band. Instead, he was homozygous for a 424 bp fragment, which was also present in all heterozygous family members harboring the K372R amino acid exchange (Fig. 4A). Direct sequencing from cDNA confirmed skipping of exon 10 (116 bp) in all individuals with the mutated allele. This skipping leads to a frame shift and a premature STOP codon of the remaining open reading frame, abolishing the template for the α- and the β-chain portions of the open reading frame (Fig. 4B). Thus, the sequence alteration could represent a noncanonical splicing mutation that alters the efficiency of an ESE, resulting in the loss of exon 10. Another explanation for this loss of splicing mutation is that the nucleotide substitution is close to the 5’ exon/intron junction and...

FIGURE 4. hC5 RT-PCR from exons 9–12. In all individuals having at least one normal allele we detected the expected normal 540 bp band. The propositus is homozygous for a 424-bp fragment, which was also present in all heterozygous family members harboring the K372R amino acid exchange (A). Direct sequencing from cDNA confirmed skipping of exon 10 (116 bp) in all individuals with the mutated allele (B), which results in a frame shift and a premature STOP codon of the remaining open reading frame.
may consequently disrupt the recognition of the 5′ splice site. Applying a maximum entropy model of splice sites, the mutation reduces the agreement of the C5 5′ splice site with the consensus (MAXENT score 7.24 is reduced to 2.55 with the identified mutation). However, this change does not appear to invalidate aggg tagat as a 5′ splice site, as this exact sequence is used as the 5′ splice site for exon 4 of the human topoisomerase (DNA) II binding protein 1 (TOPBP1) (NM 007027).

**Discussion**

Extended immunohistochemical and functional analyses led to the identification of a novel molecular basis for a C5 deficiency. The patient described herein, suffering from recurrent infections, had an almost undetectable serum level of C5. In both consanguineous parents and in one of two siblings, a markedly reduced concentration of serum C5 was observed, suggesting a heterozygous state for a mutant hC5 gene. To prove this hypothesis, the sequence of the C5 gene was determined in each family member. Surprisingly, direct sequencing of all coding exons, flanking regions and promoter revealed an apparent trivial mutation that could not explain the almost complete lack of the C5 protein. The nucleotide substitution predicted the replacement of the normal lysine with an arginine in codon 372. This is a conserved change, since both amino acids belong to the group of alkaline residues. This sequence alteration was homozygous in the patient and heterozygous in those family members with reduced serum C5. It was therefore tempting to speculate that if not causative the result was tightly linked to the defect, even though the identified mutation seemed to have no obvious impact on the normal splice site. Recently, it was found that exonic sequences as ESEs regulate the splicing process by facilitating recognition of exonic and intronic splice sequences. In our case, the wild-type sequence ATCAAG is eliminated by a single nucleotide substitution and a new putative ESE is created (CATCAG). However, the enrichment profiles of the wild-type hexamers suggest that the ATCAAG may be a “stronger” or “more probable” ESE than CATCAG. It is interesting to note that the hexamer ATCAAG (and not CATCAG) is significantly conserved according to a recent analysis of the frequency of ESE disruption by single nucleotide polymorphisms (SNPs) (W. Fairbrother, unpublished observation). As natural selection removes deleterious mutations from the population, variations that persist as SNPs are largely neutral and appear to “avoid” functional elements, such as ESEs. An analysis of SNPs and ESEs revealed that both sequences, CATCAG and ATCAAG, are not affected by SNPs (W. Fairbrother, unpublished observation). However, the selective pressure on ATCAAG is 2-fold greater than the selective pressure on CATCAG. In other words, if all genomic occurrences of ESE hexamers (in exons) are considered, the frequency of an interruption by a SNP is 18.4% less than expected for CATCAG while ATCAAG is interrupted 38.3% less than expected. Furthermore, when repeating the RESCUE-ESE protocol in the mouse, ATC AAG is present in both the mouse and human ESE set, while CATCAG is only present in the human set. Taken together, these data suggest that ATCAAG is a “stronger” or “more probable” ESE than CATCAG. This finding is consistent with the observed loss of function, when CATCAG is mutated to CATCAGG.

There is certain evidence that ESEs are comprised of diverse sequences and occur frequently within exons. Ominously, these latter studies predict that many human genetic diseases linked to mutations within exons might be caused by the inactivation of ESEs (21). If the ESE alteration has the proposed effect, it should result in abnormal splicing of the affected exon 10 in the mutated allele. We therefore sequenced cDNA directly which was transcribed from lymphocyte RNA and found skipping of exon 10 in all family members who carry the mutated allele. Because this mutation leads to a frame shift and a premature stop codon after 336 aa, the reduced level of C5 in the heterozygous carriers and the almost complete C5 deficiency in the patient can be explained. To rule out the possibility of intronic mutations, which may also affect splicing, we sequenced the branching site in intron 9. No sequence alteration was detected. This again argues for the ESE alteration being the mechanism for the C5 protein deficiency. As mutations that cause exon skipping are, in general, harmful, they are eliminated by natural selection. To prove that the A to G transition is such an ESE mutation we sequenced exon 10 of 50 healthy blood donors of the same ethnic background and did not detect the sequence alteration in any individual (data not shown). These findings strongly suggest that the C5 deficiency reported here is due to A1115G and that our observation does not reflect a common polymorphism in the Turkish population.

The position of the sequence alteration could also indicate that the mutation, rather than affecting the efficiency of an ESE, disrupts the recognition of the 5′ splice site. Indeed, the mutation does reduce the agreement of the C5 exon 10 5′ splice site according to the maximum entropy model of splice sites. However, the altered 5′ splice site seems to be still efficient, since the identical sequence motif (aggg tagat) is the functional 5′ splice site for exon 4 of TOPBP1. This again argues for the nucleotide substitution being deleterious due to subsequent ESE alteration.

A growing number of disease-causing mutations have been shown to inactivate ESEs, thereby causing exon skipping. Well known ESE mutations affect the genes BRCA2, GH1, and CFTR. Many of the deleterious BRCA2 mutations have an effect on the splicing of the gene transcript. As in our case, they are typically located close to intron/exon boundaries. Fackenthal and coworkers (22) systematically examined several BRCA2 mutations for potential ESE disruption mutations and identified a Thr2722-to-Arg mutation. It segregated with the affected individuals of a family with breast cancer and disrupted three potential ESE sites. This mutation caused deleterious protein truncation due to exon skipping and suggested its analysis as a potentially useful method to elucidate the clinical significance of a subset of the hitherto unclassified variants of BRCA1 and BRCA2. Furthermore, in growth hormone type 1 (GH1) deficiency, three different mutations have been described (23, 24, 25) which are not located at the 5′ splice site of an intron but alter splicing of GH1. All three mutations reside within sequences that resemble exonic and intronic splicing enhancers. Ryther and coworkers (26) showed that these mutations cause splicing failures due to a defect in exon definition and that splicing enhancer mutations which weaken the recognition of exon 3 produce variable amounts of the dominant-negative 17.5 kDa GH1 isoform. In a study presented by Aznarez et al. (27), the consequence of two cystic fibrosis (CF) disease-causing mutations, E656X and 2108delA, was analyzed with respect to the function of a putative ESE in exon 13 of the CF transmembrane conductance regulator (CFTR) gene. Using minigene constructs, it was demonstrated that both mutations caused aberrant splicing in a predicted manner supporting a role for the putative ESE sequence in pre-mRNA splicing. They concluded that the severity of CF could be modulated by changes in the fidelity of CFTR pre-mRNA splicing. Thus, in all three genes as well as in the current case, a particular single base exchange in an exon causes inappropriate skipping by inactivation of a critical ESE motif.

In this report we present the first case of a homozygous mutation in the hC5 gene, leading to an almost complete complement C5 deficiency. We demonstrate that an initially inconsiderable sequence alteration results in exon skipping by affecting an ESE, a mechanism that will shed a new light on the role of putative silent/
conservative mutations in disease-associated genes. Our findings indicate that putative polymorphic sequence alterations which segregate with defects, should be reviewed in this respect. Whether such noncanonical ESE mutations may explain variable phenotypes in other diseases, as it was suggested by Aznarez and co-workers (27), needs to be investigated.

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References