

A novel mutation in the *CYBB* gene resulting in an unexpected pattern of exon skipping and chronic granulomatous disease

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Abstract

Chronic granulomatous disease is a rare inherited disorder caused by non-existent or severely decreased phagocyte superoxide production that results in a severe defect in host defense and consequent predisposition to microbial infection. The enzyme responsible for superoxide production, NADPH oxidase, involves at least five components. An absence of, or a defect in, any one of four of these proteins (p47^{phox}, p67^{phox}, p22^{phox} and gp91^{phox}) gives rise to the known types of chronic granulomatous disease. The most common form of inheritance is X-linked and is due to mutations in the *CYBB* gene that encodes gp91^{phox}, the large subunit of flavocytochrome *b*, the terminal electron donor of the oxidase. We have recently reported a large number of mutations in this gene revealing a broad range of defects, including large and small deletions, and frameshift, nonsense, missense, splice region and regulatory region mutations. Here we report a patient who has an unusual type of mutation that results in the generation of a ‘pseudo-exon’ in the gp91^{phox} mRNA and an unexpected pattern of splicing. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Chronic granulomatous disease (CGD) is a rare inherited disorder in which phagocytes (neutrophils, eosinophils, macrophages and monocytes) fail to undergo a full respiratory burst when stimulated [1,2]. The products of the respiratory burst, superoxide (O₂[–]) and the reactive oxygen species derived from it, are critical to the effective destruction of microorganisms by these cells. Consistent with this

defect, CGD patients suffer from severe, recurrent bacterial and fungal infections. In most cases, patients present early in life with multiple pyogenic infections, but in less severe cases (where there is residual NADPH oxidase activity), the diagnosis may occur in adolescence or early adulthood.

The enzyme responsible for the respiratory burst, NADPH oxidase, is a complex, multicomponent enzyme consisting of at least five components (reviewed by DeLeo and Quinn [3]). Together, these proteins form a transmembrane electron transfer system, capable of oxidizing cytosolic NADPH and reducing external molecular oxygen to form O₂[–]. This enzyme is located in the plasma and specific granule mem-

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branes of the cells and is incorporated into the phagosome during phagocytosis, thereby directing O_2^- into the vacuole containing the engulfed microorganism. Defects in any one of four of the NADPH oxidase proteins (p47^{phox}, p67^{phox}, p22^{phox} or gp91^{phox}) give rise to the known forms of CGD. The most common form of the disease is X-linked, and results from mutations in the gp91^{phox} gene (*CYBB*) that codes for the β -subunit of flavocytochrome b_{-245} , the core of NADPH oxidase. The 30-kb *CYBB* gene consists of 13 exons and is located at Xp21.1. Our studies and those of others have revealed a broad distribution of defects in this gene in patients with CGD [4–7]. These include small and large deletions, insertions, and point mutations that lead to premature stop codons, amino acid substitutions, splice site defects and, rarely, mutations in the 5' regulatory region. In most cases, the amount of flavocytochrome b_{-245} is diminished or undetectable in the patient's phagocytes. As part of our continuing characterization of mutations causing CGD, we recently evaluated a patient with the X-linked form of the disease who had an unusual type of splicing defect.

2. Materials and methods

2.1. Patient

The patient is a 52-year-old male with a history of recurrent infections from the age of 14 months. He was diagnosed with CGD at age 24 years. Blood samples were obtained from the patient and family members following the procedures approved by the Human Subjects Committee of The Scripps Research Institute. Clinical diagnosis of CGD and carrier status were confirmed by spectrophotometric assay of the superoxide dismutase-inhibitable rate of cytochrome *c* reduction, and by flow cytometry using dihydrorhodamine as an indicator as previously described [7]. Biochemical analysis of the patient's neutrophils showed both an absence of flavocytochrome b_{-245} by spectroscopy and the absence of its two protein components (gp91^{phox} and p22^{phox}) by immunoblot. His family history is highly suggestive of X-linked inheritance, and his mother and sister have been identified as carriers by the NBT slide test [8].

2.2. Single-strand conformation polymorphism (SSCP) analysis

Genomic DNA was extracted from peripheral blood leukocytes [9]. DNA was analyzed by SSCP [10,11] utilizing two-stage PCR with 18–27-bp oligonucleotide primers and 100 μ g of genomic DNA, followed by nested PCR with 33 P end-labeled primers [5]. (Sequences available on request). The resultant products were subjected to PAGE and detected by autoradiography. SSCP analysis was performed on the 5' flanking region and all 13 exons of the *CYBB* gene.

2.3. DNA sequencing

DNA sequencing of genomic DNA was performed as described previously [5] using DNA primers designed to amplify the 5' flanking region and each of the 13 exons of the *CYBB* gene from 382 bp 5' of the coding region to 26 bp 3' of exon 13. The Sanger dideoxy sequencing method was used.

2.4. mRNA analysis

mRNA was analyzed using RNA isolated from whole blood using the RNeasy Kit (Qiagen). cDNA was prepared using RT PCR (Life Technology cDNA kit) using a forward primer from exon 1 and a reverse primer from exon 13. The cDNA products were visualized on agarose gels by staining with ethidium bromide.

3. Results

Analysis by SSCP revealed no abnormalities in the 5' flanking region or any of the 13 exons of the *CYBB* gene. In our previous experience, only seven of 131 kindreds have had mutations undetectable by SSCP [5], and these were subsequently identified by sequencing the entire set of PCR amplicons. We therefore undertook sequencing of all the exonic regions of genomic DNA, including 382 bp upstream of the coding region and 26 bp 3' of exon 13. Again, no abnormalities were found.

To investigate the possibility that intronic abnormalities within the *CYBB* gene result in aberrant

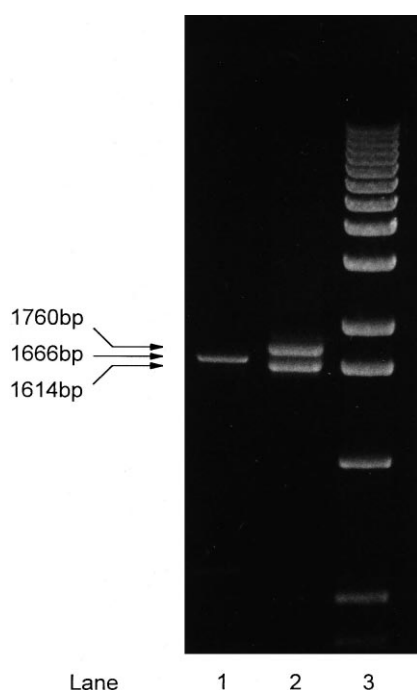


Fig. 1. Two abnormal mRNA species of approximately equal abundance are present in the patient. Analysis of mRNA transcripts of the *CYBB* gene was performed on agarose gels as described in Section 2. Lane 1, normal cDNA, 1666 bp; lane 2, patient cDNA; upper band ~1750 bp, lower band ~1600 bp; lane 3, markers.

mRNA transcripts, we analyzed cDNA (prepared from mRNA isolated from whole blood as described in Section 2) on agarose gels (Fig. 1). In contrast to the normal control (lane 1), the patient's sample contained two cDNA products of approximately equal abundance (lane 2). One of these species was approx-

imately 50 bp smaller than the normal message (1666 bp) and the other approximately 100 bp larger. Sequencing of the two cDNA fragments isolated from the agarose gels revealed a 94-bp insertion between exons 6 and 7 in both species. The larger message was otherwise normal, but the smaller message had exon 5 deleted in addition to the 94-bp insertion. In both cases, the mRNA coded for truncated proteins.

We hypothesized that the 94-bp insertion had arisen from a splicing abnormality in intron 6. We therefore sequenced intron 6 in its entirety (2.4 kb) by directed sequencing with progressive nucleotide primers [5] which was accomplished in nine steps. This analysis disclosed that the 94 bp insertion is derived from approximately the middle of intron 6. In 9 normal controls, this sequence is preceded by **taa**, but in the patient it is preceded by **tag** (Fig. 2). This single nucleotide mutation creates a sequence with a good match to the consensus 3' (acceptor) splice site (see Section 4). The sequence following the 94-bp sequence is **gtaatc** in both normal and patient sequences, and represents a consensus 5' (donor) splice site.

4. Discussion

In the absence of mutations in the coding sequence and the lack of normal *CYBB* mRNA transcripts, we propose that the primary defect in this patient is the base pair substitution, **a** → **g** in intron 6. We believe this results in the formation of a functional 3' (acceptor) splice site within the intron. The consensus 3'

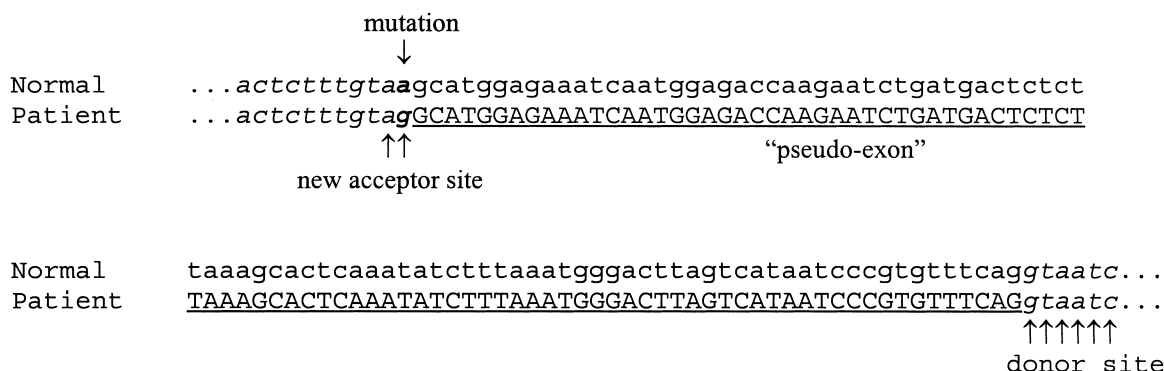


Fig. 2. Intron 6 of the patient's *CYBB* gene contains a 94-bp sequence resembling an exon. Sequencing of intron 6 (2.4 kb) was performed by gene walking in nine steps. The patient's DNA has a single base change (**a** → **g**, in bold) immediately preceding the 94-bp sequence that was incorporated into the patient's mRNA (in uppercase). The proposed donor and acceptor splice sites are marked with arrows.

A) 5' (donor) splice site consensus sequence:

-2	-1		+1	+2	+3	+4	+5	+6
A ₆₂	G ₇₇		g ₁₀₀	t ₁₀₀	a ₆₀	a ₇₄	g ₈₄	t ₅₀

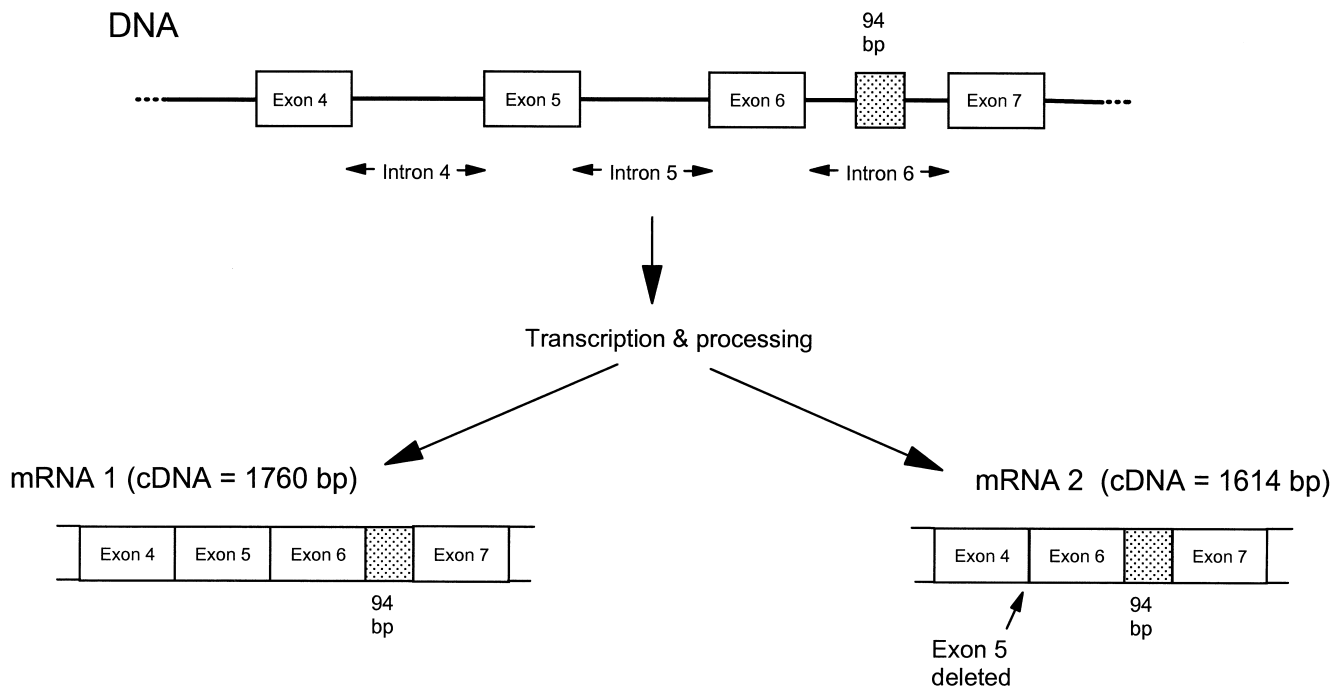
B) 3' (acceptor) splice site consensus sequence:

-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1		+1
Y ₇₈	Y ₈₁	Y ₈₃	Y ₈₉	Y ₈₅	Y ₈₂	Y ₈₁	Y ₈₆	Y ₉₁	Y ₈₇	n	c ₇₈	a ₁₀₀	g ₁₀₀		G ₅₅

Fig. 3. Consensus sequence elements necessary for correct splicing. The percentage frequency of a particular nucleotide appearing at each position is indicated by subscript. The percentages are taken from [8]. y = pyrimidine, n = any base.

splice site in mammals is a run of approximately 10 pyrimidines; followed by any base (usually c); and an invariant **ag** (Fig. 3). Scoring of the patient and normal sequences by the method of Shapiro and Senapathy [12] gives an increase in likelihood of their being an acceptor site from 70.8 to 86.9 as a result of the substitution a→g. Immediately 3' of the 94 bp sequence is a consensus donor site, **gtaatc**, that is

well within the ~300-bp maximum for the length of an exon. The **a→g** mutation appears to generate a 'pseudo-exon' that is inserted between exons 6 and 7 (Fig. 4, mRNA 1). This 'pseudo-exon' fits the loosely conserved consensus sequence for exons, starting with G and ending in AG. Due to the incorporation of this 94-bp sequence, an mRNA species ~100 nucleotides larger than normal is generated (as



(Normal mRNA = 1725 nucleotides, cDNA = 1666bp)

Fig. 4. Generation of the two abnormal mRNA species in the CGD patient. As described in Section 4, the mutation in intron 6 leads to the insertion of the 94-bp intronic sequence between exons 6 and 7 (mRNA 1). Approximately half the time, the insertion causes another splicing error which results in exon 5 being spliced out (mRNA 2).

shown in Fig. 2). Unexpectedly, approximately half the time, this new abnormal message results in the splicing-out of exon 5 (146 bp), presumably due to conformational restraints imposed by the 94-bp insertion. This leads to the second mRNA species, approximately 50 nucleotides smaller than normal (Fig. 4, mRNA 2). Searching of the Aberrant Splicing Database at <http://cookie.imcb.osaka-u.ac.jp/nakai/asdb.html> failed to find a similar type of mRNA editing error. There are several previous reports of analogous insertions involving *Alu* sequences, but, to the best of our knowledge, this type of event has not been previously reported for non-*Alu* sequences. The generation of novel ‘pseudo-exons’ by this kind of process could provide an important mechanism of gene evolution, as well as causing mutations that lead to inherited diseases.

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