A Mutation Located at the 5′ Splice Junction Sequence of Intron 3 in the p67\textsubscript{phox} Gene Causes the Lack of p67\textsubscript{phox} mRNA in a Patient With Chronic Granulomatous Disease

By Laurence Cohen Tanugi-Cholley, Jean-Paul Issartel, Joël Lunardi, Fernand Freycon, Françoise Morel, and Pierre V. Vignais

Chronic granulomatous disease (CGD) is due to a functional defect of the O₂−-generating NADPH oxidase of neutrophils. Mutations resulting in CGD have been shown to occur in only four genes, thus identifying the main components of the oxidase complex, namely the two subunits of a membrane-bound cytochrome b and two cytosolic factors of activation of 67 kD (p67\textsubscript{phox}) and 47 kD (p47\textsubscript{phox}). The present study deals with the biochemical and genetic analysis of the defect in a patient suffering from a p67\textsubscript{phox}-deficient form of CGD. The p67\textsubscript{phox} deficiency was ascertained by immunocytochemistry and the ability of recombinant p67\textsubscript{phox} to restore NADPH oxidase activity using a cell-free system of oxidase activation. The cellular extracts from the proband contained no p67\textsubscript{phox} protein and no p67\textsubscript{phox} mRNA when assayed by Western and Northern blot analysis. However, reverse transcription of mRNA and subsequent cDNA amplification by polymerase chain reaction using specific p67\textsubscript{phox} primers showed that trace amounts of a p67\textsubscript{phox} mRNA deleted for exon 3 were synthesized in the patient immortalized B lymphocytes. Sequence analysis of the genomic DNA showed a T-to-C transition at position +2 of intron 3. This point mutation in the consensus 5′ splice site of the intron 3 was probably responsible for lack of accumulation of mRNA and also for the skipping of exon 3 detected in the few mRNA molecules that escaped cellular degradation.

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MATERIALS AND METHODS

Case. The patient (GZ), the 19-year-old daughter of consanguineous parents from North Africa, has a typical history of recurrent lymphadenitis, osteomyelitis, and abscesses. The diagnosis of CGD was based on the absence of nitroblue tetrazolium (NBT) reduction by the neutrophils of the patient. The consanguineous parents did not suffer from this disease. To facilitate biochemical and genetic investigations of the molecular defects resulting in CGD, B lymphocytes from this patient were immortalized by infection with EBV.

Biological material. The preparation of human neutrophils and B lymphocytes infected with the B95-8 strain of EBV (EBV-B lymphocytes), as well as that of cytosol and membranes has been previously described.

Cell-free system of oxidase activation. The cell-free activation system contained membranes and cytosol from neutrophils or EBV-B lymphocytes, supplemented with GTP, MgCl₂, and arachidonic acid. The rate of production of O₂− was followed by the supernatase-dismutase (SOD) inhibitable reduction of cytochrome c.

Complementation of cytosol from p67-deficient EBV-B lymphocytes by recombinant p67\textsubscript{phox}. Recombinant p67\textsubscript{phox} expressed in the baculovirus-SF9 insect cell system was a gift from Drs M.C. Daugherty and A. Fuchs. The cDNA encoding p67\textsubscript{phox} was cloned in the baculovirus transfer vector phiac Bac III under control of the polyhedrin promoter.
**SPLICE JUNCTION MUTATION IN A P67(-) CGD PATIENT**

**Spectral analysis of cytochrome b_{558}.** Spectrophotometric measurements were made on sonicated neutrophils and EBV-B lymphocytes suspended in 0.2 M sucrose buffered with 0.05 M MOPS (pH 7.3). Reduced against oxidized difference spectra were recorded at 77K with a double-beam Perkin Elmer 557 spectrophotometer (Perkin Elmer, Norwalk, CT).

**Immunoblotting.** Cytosolic fractions (300 μg protein from EBV-B lymphocyte homogenate) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoresed onto a nitrocellulose membrane and immunodetected with specific antisera directed against the p67\textsuperscript{phox} and the p47\textsuperscript{phox} cytosolic factors of human neutrophils.\textsuperscript{20} Two types of polyclonal anti-p67\textsuperscript{phox} antibodies were used. The first type was raised against the synthetic peptide extending from amino acid 512 to the C-terminus, the second one was an affinity immunopurified polyclonal antibody directed against the N-terminus (residues 1 to 257) of p67\textsuperscript{phox} and produced in *Escherichia coli* as a glutathione transferase hybrid. The latter antibody was kindly provided by Prof A. N. Segal.

**Isolation of RNA and DNA.** Freshly harvested EBV-B lymphocytes were disrupted in 4 M guanidine thiocyanate. The homogenate was layered on a cushion of 5.7 M cesium chloride and total RNA obtained as a pellet after centrifugation.\textsuperscript{26} Genomic DNA was isolated from EBV-B lymphocyte following classical procedures.

**Northern blot analysis.** Purified RNA (20 μg) was size fractionated by vertical electrophoresis at 130 V for 4 to 5 hours, using a 1.45% (w/v) agarose gel containing 2.2 M formaldehyde. RNA species were visualized by ethidium bromide staining, transferred onto a nylon membrane (N-Hybond; Amersham, Little Chalfont, UK) by capillarity, and then UV cross-linked to the membrane. Hybridization was performed with a 1.6-kbp native ~67\textsuperscript{C}-~67\textsuperscript{P} cDNA probe extending from base 19 to base 1633, numbered from the start of translation. A glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe was used as control. Nucleic acid probes were radiolabeled using the T7 QuickPrime Kit (Pharmacia, Uppsala, Sweden) and (α\textsuperscript{32P}) dCTP (Amersham). After hybridization and extensive washing, membranes were autoradiographed at −70°C using intensifying screens.

**Amplification of cDNA or genomic DNA.** mRNA was specifically detected by reverse transcription followed by polymerase chain reaction (RT-PCR).\textsuperscript{27} Single-strand cDNA was synthesized using a kit from Pharmacia; the reverse transcriptase was primed with a random hexamucleotide mixture provided by the manufacturer. Subsequent amplification of specific DNA fragments was performed with oligonucleotide primers selected for their ability to target the 2.2-kbp sequence of the cDNA coding for ~67\textsuperscript{C} (Table 1). Amplification of specific regions of the genomic DNA also required the set of primers described in Table 1. Oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems Inc, Foster City, CA).

**Cloning and sequencing of the amplified fragments.** After amplification, the DNA fragments were size-fractionated on Seaplaque or Seakem agarose gels (FMC Bioproducts, Rockland, ME). Fragments of interest were excised from the gels, then extracted and purified using a silica matrix-based procedure (QIAGEN; Diagen, Hilden, Germany). Purified cDNA fragments were ligated directly into T-vector derived from pBluescript II-SK\textsuperscript{-} phagemids (Stratagene, La Jolla, CA) that had been digested with EcoRV and tailed with a single 3’T overhang.\textsuperscript{28} A fraction of the ligation mixture was used to transform competent cells of XLblue E coli (Stratagene). Resulting clones were subsequently grown and infected by the VCSM13 phage helper to produce single-strand DNA phagemids following the protocol recommended by Stratagene. Single-strand DNA templates were sequenced by the dideoxy chain-termination method\textsuperscript{29} using a T7 DNA polymerase sequencing kit (Pharmacia) and (α\textsuperscript{32P}) dATP (Amersham) together with appropriate primers complementary to the sequence of the phagemid vector. In all cases, sequences were verified by sequencing both strands of the cloned fragments. The sequence products were subjected to electrophoresis in 6% polyacrylamide gels at 46 W. After drying, gels were autoradiographed overnight at room temperature. Computer analysis of the sequencing data was performed with the help of DNAstar software (DNASTAR, Madison, WI).

**RESULTS**

**Biochemical characterization of the defect.** The patient, GZ, was diagnosed as suffering from CGD on the basis of absence of O\textsubscript{2}, production by blood neutrophils stimulated with *Staphylococcus aureus*, as measured by chemiluminescence and the NBT test. From the spectral properties of the neutrophil membranes it was calculated that the cytochrome b\textsubscript{558} concentration per milligram of protein of the whole neutrophil homogenate in the patient membranes was in the range of 100 to 110 pmol, a value corresponding to the lower limit of values found in healthy subjects used as control (between 115 to 265 pmol/mg protein of the homogenate). It was inferred that the functional defect was not caused by the lack of the redox component of the oxidase complex, but probably resulted from the absence of one of the cytosolic factors. Evidence for a functional defect in a cytosolic factor was provided by an experiment performed with an heterologous cell-free system of oxidase activation in which cytosol and membrane fractions from human neutrophils were complemented with membrane and cytosol fractions from GZ EBV-B lymphocytes, respectively. GZ EBV-B lymphocytes failed to support normal oxidase activation when assayed in the presence of control neutrophil membranes, as shown by the very low rate of O\textsubscript{2} production (16 ± 2 nmol of O\textsubscript{2} per minute and per mg of membrane protein) compared to the high rate values obtained with cytosol of control EBV-B lymphocytes and membranes of control neutrophils (450 ± 33 nmol of O\textsubscript{2} per minute and per mg of membrane protein) (Table 2). A homologous mixture consisting of control human neutrophil membranes mixed with control human neutrophil cytosol was able to produce O\textsubscript{2} at a rate close to 1,200 nmol of O\textsubscript{2} per minute and per mg of protein (Table 2). These results showed that the defective component of the oxidase complex responsible for the CGD of patient GZ was a cytosolic factor of oxidase activation.

Earlier studies performed with polyclonal antibodies directed against the bovine p67\textsuperscript{phox} protein suggested that patient GZ was deficient in p67\textsuperscript{phox}, but not in p47\textsuperscript{phox}. These results were corroborated by Western blot using two different antibodies directed against the C-terminus and the N-terminus of the human p67\textsuperscript{phox} protein, as illustrated in Fig 1, panels 1 and 2, respectively. In both cases, neither the whole 67-kD protein nor a truncated fragment related to p67\textsuperscript{phox} were shown in cytosol of GZ EBV-B lymphocytes. The p67\textsuperscript{phox} deficiency in GZ EBV-B lymphocytes was confirmed by in vitro complementation of the cell-free system conducted first with cytosol of human neutrophils containing a normal amount of p67\textsuperscript{phox} but lacking p47\textsuperscript{phox} and more recently with recombinant p67\textsuperscript{phox} (Fig 2). As illustrated in the dose-effect curve of Fig 2, in a cell-free system consisting of normal neutrophil membranes and cytosol from GZ-EBV-
B lymphocytes, oxidative activation was restored up to 80% of the maximum on addition of cytosol of S9 cells infected by baculovirus and surexpressing p67^{box}. In contrast, when cytosol from control S9 cells was added to the cell-free system, oxidative activation remained defective, pointing to the specificity of the restoration of the GZ defect by recombinant p67^{box}.

Taken together, these results provided definite evidence that patient GZ was afflicted with the very rare form of the autosomal recessive CGD associated with the absence of the p67^{box} component.

**Genetic analysis of the defect.** Absence of p67^{box} in EBV-B lymphocytes from GZ patient could be caused by a transcriptional or translational defect. Putative deficiency at the transcriptional level was investigated by Northern blot analysis. Total cellular RNA from GZ EBV-B lymphocytes (Fig 3, panel 1). Under similar conditions, no radioactive signal either at 2 kbp or below was found with RNA from GZ EBV-B lymphocytes (Fig 3, panel 1). This result indicated that the RNA responsible for the synthesis of p67^{box} was absent in patient GZ. The quality and the quantity of GZ RNA samples was ascertained by the positive hybridization obtained with a cDNA probe that was able to target the glyceraldehyde phosphate dehydrogenase transcript (Fig 3, panel 2). Similar results were obtained with four different materials and Methods, they were hybridized with various ^32P-labeled cDNA probes (Fig 3). Using a fragment of normal p67^{box} cDNA extending from nucleotide −19 to 1603 and corresponding to the translatable fragment as a probe, an intense radioactive signal at about 2 kbp was observed with RNA prepared from normal EBV-B lymphocytes in agreement with the predicted size of a p67^{box} gene transcript (Fig 3, panel 1). Under similar conditions, no radioactive signal either at 2 kbp or below was found with RNA from GZ EBV-B lymphocytes (Fig 3, panel 1). This result indicated that the RNA responsible for the synthesis of p67^{box} was absent in patient GZ. The quality and the quantity of GZ RNA samples was ascertained by the positive hybridization obtained with a cDNA probe that was able to target the glyceraldehyde phosphate dehydrogenase transcript (Fig 3, panel 2). Similar results were obtained with four different

### Table 1. PCR Oligonucleotide Primers Used in This Study

<table>
<thead>
<tr>
<th>cDNA primers</th>
<th>Genomic primers</th>
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<tr>
<td>1. F2</td>
<td>5'-CTCTAGTTTTCTACTAATC-3'</td>
</tr>
<tr>
<td>2. F3</td>
<td>5'-AGGGATGCTCTACTACCAAGAG-3'</td>
</tr>
<tr>
<td>3. R2*</td>
<td>5'-GCTTTGATTGTGAAGATCCT-3'</td>
</tr>
<tr>
<td>4. R4*</td>
<td>5'-TTCCAGAGCCCTAGAATCT-3'</td>
</tr>
<tr>
<td>5. R5*</td>
<td>5'-GCTGAATCAAAGCTTCT-3'</td>
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* Antisense primer.

### Table 2. Determination of the Elicited Oxidase Activity in Homologous and Heterologous Cell-Free Systems of Oxidase Activation

<table>
<thead>
<tr>
<th>Origin of Membranes and Cytosols</th>
<th>O2 Production (nmol min⁻¹ mg⁻¹ Membrane Protein)</th>
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<tbody>
<tr>
<td>1. Human neutrophil membranes (N) + Human neutrophil cytosol (N)</td>
<td>1.179 ± 131 (n = 6)</td>
</tr>
<tr>
<td>2. Human neutrophil membranes (N) + EBV-B lymphocyte cytosol (N)</td>
<td>450 ± 33 (n = 13)</td>
</tr>
<tr>
<td>3. Human neutrophil membranes (N) + EBV-B lymphocyte cytosol (GZ)</td>
<td>16 ± 2 (n = 10)</td>
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The cell-free system of oxidative activation included membranes (30 μg protein) and cytosol (300 μg protein) from neutrophils or EBV-B lymphocytes from healthy adults (N) and GZ patient (GZ), 5 mmol/L MgCl₂, 40 μmol/L GTP-γ-S, and an optimal concentration of arachidonic acid in a final volume of 100 μL. The rate of O₂ production was determined as the rate of O₂ inhibitable cytochrome c reduction. It was expressed as nanomoles per minute and per milligram of membrane protein ± SD. Number of experiments (n) are indicated in parentheses.

**Fig 1.** Western blot analysis of cytosol fractions from EBV-B lymphocytes from healthy adult (N) and GZ patient (GZ) with anti-p67^{box} antibodies. Cytosol fractions were subjected to SDS-PAGE. After transfer onto nitrocellulose membrane, p67^{box} was probed with p67-antibodies. 1, Antipeptide-antibodies against the C-ter part of p67^{box}, 2, antibodies directed against the N-ter part of the protein. The immune complexes were detected with alkaline phosphatase-conjugated goat-antirabbit IgG and NBT-5-bromo-4-chloro-3-indoly-phosphate (NBT-BCIP) as substrate.
RNA preparations issued from four different preparations of GZ EBV-B lymphocytes. Hybridization with a p47phox cDNA probe gave consistent signals both with the control and the patient’s RNA (not shown).

As minute amounts of mRNA could have escaped detection by Northern blot analysis, we set up an amplification by RT-PCR to check whether the p67phox gene was transcribed at a very low level in GZ EBV-B lymphocytes. Total cellular RNA was first reverse-transcribed and the resulting single-strand cDNA was submitted to DNA specific amplification using a thermal cycling system with pairs of p67phox-specific oligonucleotide primers. These primers were used to amplify a number of fragments encompassing the whole p67phox cDNA sequence. Using RNA from GZ EBV-B lymphocytes as starting material, RT-PCR experiments allowed the amplification of fragments that proved to be p67phox cDNA fragments, including a PCR product generated through the use of the F2 and R2 primers which corresponded to the full length of the coding deleted sequence. This result obtained through the very sensitive RT-PCR approach indicated that GZ EBV-B lymphocytes contained p67phox mRNA, but this mRNA was present in trace amounts and so escaped detection by Northern blot analysis. As shown in Fig 4, when primers were chosen to amplify the sequence extending from base –19 to base 301, the cDNA fragment was generated with a correct size in the control experiment, whereas in the case of GZ RNA, the generated cDNA fragment appeared shorter by about 80 bp. All amplified fragments (control and patient) encompassing the whole p67phox cDNA sequence were cloned and sequenced. The sequence of the cDNA fragments amplified in the control samples proved to be 100% identical to the published sequence. On the contrary, the GZ cDNA sequence showed an 83-bp deletion located between nucleotide 174 and 258 (Fig 5, A and B). As a control, cDNA amplification was performed with two other primers, one of which was designed to hybridize the deleted region (primer F3 in Table 1), the other being primer R4 (Table 1). As expected, mRNA from normal EBV-B lymphocytes led to the amplification of a 497-bp fragment that was not detected when mRNAs from the GZ EBV-B lymphocytes were used as templates. This experiment clearly indicates that only p67phox-deleted mRNA was present in the GZ EBV-B lymphocytes. No other mutation was detected upstream or downstream of this deletion in the cDNA, excluding a second allelic mutation. According to the exonic organization of the p67phox gene re-
The sequence of PCR steps was as follows: an initial denaturation step of 94°C for 4 minutes, then 30 cycles with 1 minute denaturation at 94°C, 1 minute annealing at 48°C, 1 minute extension at 72°C followed by a 7-minute final extension at 72°C. One tenth of the preparation was subjected to electrophoresis in a 1.5% agarose gel containing ethidium bromide. Values indicated correspond to fragment size of marker DNA loaded in lane M.

The consequence is a translational frameshift after codon 58. Interestingly, analysis of the patient’s genomic DNA showed that exon 3 in the p67phox gene was not deleted. No non-sense mutation was found in exon 3 so that skipping of exon 3 is not related to the presence of a premature stop codon, which could lead either to exon skipping as recently reported in cystic fibrosis transmembrane conductance regulator (CFTR) transcript28 or to reduced accumulation of mRNA as described for the β-globin transcript29 or to combination of both phenomena, i.e., exon skipping and lower accumulation of mRNA as in the case of the fibrillin trans-

DISCUSSION

As evidenced by the immunologic investigations performed with EBV-B lymphocytes from a p67phox-deficient patient (GZ), the p67phox protein was completely absent from lymphocyte cytosol. When assayed in a cell-free system of oxidase activation, the cytosolic fraction prepared from the EBV-B lymphocytes proved to be inefficient in promoting NADPH-dependent O2− generation. The oxidase activity was restored by addition of cytosol from CGD neutrophils lacking p47phox, but containing a normal amount of p67phox,21 and by addition of recombinant p67phox expressed in the Sf9 cell baculovirus system (Fig 2). This observation clearly indicates that aside from the absence of the p67phox protein, there was no other pathologic impairment in the 02−-generating NADPH oxidase of GZ cells. Northern blot and RT-PCR analysis showed additional interesting features concerning this genetic defect. (1) The p67phox mRNA was undetectable by hybridization with specific probes. (2) The few p67phox mRNA molecules detected in the extracts of the EBV-B lymphocytes by use of the highly sensitive RT-PCR were found to be deleted. Sequence analysis of the amplified cDNA showed that the deleted sequence in the mRNA species was located between base 174 and base 258. In other words, exon 3 was skipped over during the course of transcription or mRNA processing. As a consequence of abnormal splicing, a translational −1 frameshift was predicted to occur after codon 58, generating a premature TGA stop codon at position 60. So, both the limited amount of p67phox transcript and the putative translation of a truncated p67phox protein combine to make the patient cells completely devoid of functional p67phox protein.
Fig 5. (A) Sequence analysis of amplified p67\textsuperscript{phox} cDNA. cDNA fragments were amplified by PCR and cloned into a Bluescript-SK+ T-vector (see Materials and Methods). Sequence analysis was performed by the dideoxy-chain termination method. The sequence obtained from patient G2 is on the left (GZ), the control sequence is shown on the right (N). The arrows point to the mutations. The sequence shown in lowercase letters was read on the complementary strand and the coding sequence is shown in uppercase letters. (B) Alignment of the sequence of the normal cDNA (N) from nucleotides 154 to 187 with the sequence of deleted cDNA from bases 154 to 187 from G2 patient (GZ). Translation products of both cDNA fragments are given to illustrate the -1 translation frameshift resulting into the generation of a stop codon (X) in G2 coding sequence. Deleted nucleotides are indicated by dashes. Heptanucleotide repeats flanking the edges of the deletion are underlined.

The sequence in the vicinity of exon 3 was entirely identical to that published by Kenney et al\textsuperscript{19} except that T was substituted by C at position +2 of the consensus 5' splice site at the exon 3-intron 3 boundary. No other mutation was found either in the promoter region or in the sequence of the GZ p67\textsuperscript{phox} cDNA. On the other hand, restriction analysis of the genomic DNA did not show any detectable deletion or polymorphism in one of the alleles (not shown). Furthermore, because of the consanguinity of her parents, it is likely that GZ is homozygous for the mutated p67\textsuperscript{phox} gene. Based on these different considerations, it is inferred that the muta-
tion in the splice site at the beginning of intron 3 is the only one responsible for the low accumulation of \( p67^{phox} \) mRNA and the skipping of exon 3.

Repetition of the heptanucleotide motif (CAGAGAA) at both the 3' end of exon 2 and the 3' end of exon 3 (Fig 5B) is noteworthy. However, the assumption that these sequences might be involved in any transcriptional defect leading to the skipping of exon 3 is speculative at present. The fact that substitution of T by C at the very beginning of intron 3 causes skipping of the upstream exon is supported by previous studies in which point mutations were detected in the 5' splice sites of introns located downstream of skipped exons.35-38 In cases of CGD with mutations located in the subunits of cytochrome b_55, nucleotide transition or transversion at positions +1 or +2 of the 5' splice sites of introns have been found to result in the skipping of the immediate upstream exons.37,38 It was hypothesized that during the processing of mRNA the ribonucleoprotein particles involved in the splicing cannot bind efficiently to the 3' splice site of an intron if no ribonucleoprotein particle is interacting with the 5' splice site of the next intron.35 It has been reported that a G to A transition at position +5 of intron 4 of the human catalase gene is responsible for the skipping of exon 4 and the subsequent promotion of the degradation of this abnormal mRNA.36 Thus, the evidence that a point mutation at a 5' donor splice site in the catalase gene results in the absence of corresponding mRNA in the Japanese-type acatalasemia, clearly supports the view that the point mutation located at position 2 of intron 3 of \( p67^{phox} \) gene is directly related to the lack of accumulation of \( p67^{phox} \) mRNA.

ACKNOWLEDGMENT

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A mutation located at the 5' splice junction sequence of intron 3 in the p67phox gene causes the lack of p67phox mRNA in a patient with chronic granulomatous disease

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