Characterization of the p67\textsuperscript{pox} Gene: Genomic Organization and Restriction Fragment Length Polymorphism Analysis for Prenatal Diagnosis in Chronic Granulomatous Disease

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The genetic defect in the p67\textsuperscript{pox}-deficient form of chronic granulomatous disease (CGD) follows an autosomal recessive pattern of inheritance. When genomic DNA from normal individuals is digested with HindIII and probed with p67\textsuperscript{pox} cDNA an allelic restriction fragment length polymorphism (RFLP) of 4.0 kb or 2.3 kb is detected. We cloned and characterized the p67\textsuperscript{pox} gene using the cDNA and sequenced the exon/intron boundaries, mapping 16 exons on the 40-kb gene. The polymorphic region was then sequenced to identify the inheritance pattern of amniocentesis-derived fetal cells by genomic amplification. The proband, a 9-year-old female patient with p67\textsuperscript{pox}-deficient CGD, and her phenotypically normal mother are homozygous for the RFLP marker, whereas the father and two brothers are heterozygous. The fetus was shown to be heterozygous as well, showing it had inherited at least one normal p67\textsuperscript{pox} gene from the father and that it was predicted to have a normal phenotype. Cord blood samples at birth showed normal oxidative function. Amplification allows rapid detection of the inheritance pattern for fetal diagnosis in informative families. We report the genomic structure of p67\textsuperscript{pox} and an amplification-based method for detection of the marker on chromosome 1q25, used here for prenatal diagnosis of CGD. This is a US government work. There are no restrictions on its use.

CHRONIC GRANULOMATOUS diseases (CGDs) of childhood are a genetically heterogeneous group of inherited disorders, all resulting from an inability of phagocytic cells to produce superoxide. Typically, affected individuals are susceptible to infection with a broad range of bacteria and fungi that are less pathogenic in the normal host. Prophylactic antibiotics\textsuperscript{1} and interferon-\gamma\textsuperscript{2} have significantly reduced the infection rate for these patients, yet the morbidity of each infection can be great. Safe prenatal diagnosis of disease markers in families at risk is one key to genetic counseling.

In CGD phagocytes, the superoxide generating NADPH oxidase is disabled by the absence or defect of one of at least four constituent proteins, depending on the inherited subtype. The defect in all patients studied has been traced to one of the two membrane-associated cytochrome b$_{558}$ subunits or one of two cytosolic proteins.\textsuperscript{3,4} Several patients with autosomal recessive CGD lack the 67-kD phagocyte oxidase (p67\textsuperscript{pox}) cytosolic factor, which is required to form a functional oxidase. Recombinant p67\textsuperscript{pox} protein was shown to correct the oxidase defect in a cell-free reconstitution assay in a patient with the p67\textsuperscript{pox}-deficient form of CGD.\textsuperscript{5,6} The gene for this cytosolic factor has been mapped to chromosome 1q25 and assigned as locus symbol NCF2 (MIM no. 233710).\textsuperscript{7}

Prenatal diagnosis in CGD has relied in large part on the measurement of oxidase activity of fetal blood neutrophils sampled as early as 16 weeks gestation.\textsuperscript{8} Umbilical cord fetal-blood sampling is technically difficult, may carry a higher risk for the fetus than amniocentesis, and has the potential to give falsely positive or negative results. More accurate diagnoses can be made by analysis of sequence polymorphisms in or near the affected gene using Southern blots of fetal DNA. Markers flanking the X-CGD gene have been used to detect an affected fetus by linkage analysis.\textsuperscript{9} Restriction fragment length polymorphisms (RFLPs) have since been identified within the 91-kD cytochrome b$_{558}$ gene, which makes detection simpler in informative X-linked families.\textsuperscript{10-12} Recently a prenatal diagnosis was made from a chorionic villus biopsy in a family with X-linked disease using the polymerase chain reaction (PCR).\textsuperscript{13}

While the mutations causing the defect in p67\textsuperscript{pox} are not yet known, it has become possible to predict whether an individual from an informative family has inherited a normal gene based on a normal allelic RFLP marker that follows a Mendelian pattern of inheritance.\textsuperscript{14} Here, we report the first amniocentesis diagnosis in autosomal CGD using this technology in an informative affected family. We cloned and characterized the gene to create an exon map and sequenced the exon/intron boundaries. We were then able to determine the location and sequence of the RFLP, and to develop amplification primers that simplify the analysis of this intragenic marker on the long arm of chromosome 1.

MATERIALS AND METHODS

The use of patient material for the studies reported here was approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases.

Analysis of the p67\textsuperscript{pox} gene. Genomic clones were obtained from a normal human placental X-phage library (Stratagene, La Jolla, CA) by hybridization screening with the complete p67\textsuperscript{pox} cDNA insert as a probe. A cosmid library, constructed by partial digestion of normal human genomic DNA and ligation into the pWE15 vector (Stratagene), was also screened. Restriction sites were mapped for each clone using the primer recognition sites lo-
cated just outside the insert and inside Not I sites. Clones were completely digested with Not I and partially digested with EcoRI, HindIII, Sac I, and Xho I. Each digest was then separated in agarose using field inversion gel electrophoresis and blotted onto Nytran nylon membrane (Schleicher & Schuell, Keene, NH) according to standard methods. Hybridization of the primer to either end of the insert identifies the size of the restricted fragment for each enzyme site. Overlapping restriction maps were collated and a consensus map was obtained. Exon/intron boundaries were characterized by subcloning genomic fragments and using cDNA primers to sequence the intronic region around each exon.

The polymorphic site was localized to a 4-kb HindIII fragment at the 5' end of the gene, downstream from the last exon within this fragment, by comparing the restriction maps of the genomic clones and the Southern blots of several individuals' genomic DNA probed with the cDNA. The sequence of this region of intron 2 was confirmed by sequencing clones generated by amplification of genomic DNA from individuals homozygous for either allele 1 or allele 2. Sequence analysis was performed using the Genetics Computer Group Sequence Analysis Software Package on a Convex C240, maintained by the Division of Computer Research and Technology at the National Institutes of Health.

Proband and family history. CGD was diagnosed at age 6 months, after the proband developed Serratia marcescens cellulitis. She has had recurrent otitis media, urinary tract infections, pharyngitis, a hepatic abscess, and oral candidiasis. At the time of her mother's pregnancy studied here, the patient was 8 years old. One brother, who died at age 20 months as a result of infection, was also thought to have had CGD. The four older brothers, parents, and extended family have no history of recurrent infections. The pregnancy studied here went to term without complications.

Aminoicestasis was performed on the proband's mother as part of her normal clinical care because she was over 35 years old. Amniotic fluid was obtained by standard technique at a gestational age of 12 weeks. The cells were cultured and a portion used for this study. DNA was extracted from these cells and from peripheral blood leukocytes from several family members using routine methods. Five micrograms DNA from each individual was digested with HindIII and size fractionated on a 0.7% SeaKem GTG agarose gel (FMC BioProducts, Rockland, ME). The DNA was denatured and blotted onto Nytran. An EcoRI-Sac I fragment of the p67phox cDNA (nucleotides 1 through 843), which hybridizes to exons 1 through 9, was labeled using random primers (Boehringer Mannheim Biochemicals, Indianapolis, IN) and used as the probe. The blot was hybridized for 18 hours at 42°C, washed at high stringency, then exposed to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) for 36 hours.

p67phox gene amplification. p67phox genomic targets were amplified by PCR using upstream primer 5'-CAGTGAAGCAGAGAAAGTGAGTGG-3' and downstream primer 5'-TCTTGGAAAGCCAAAAGGAGTGC-3', corresponding to sequence from the 4-kb HindIII genomic subclone. The reaction mixture contained 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L MgCl2, 0.2 mmol/L each dNTP, 0.5 mmol/L each primer, 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT), and 100 ng genomic DNA in a volume of 100 μL. In the positive control, the template used was the cloned 4-kb genomic fragment; negative control template was the vector DNA. Temperature cycling was performed with a GeneAmp PCR System 9600 (Perkin-Elmer/Cetus, Emeryville, CA) using an initial denaturation step of 95°C for 3 minutes, then 40 cycles with 30-second denaturation at 95°C, 30-second annealing at 60°C, 30-second extension at 72°C, followed by a 5-minute final extension at 72°C. Reactions were washed on a Centricon 30 microconcentrator (Amicon, Beverly, MA) twice with 2 mL H2O. Ten percent of the reaction was digested with HindIII and electrophoresed on a 4% to 20% gradient TBE-polyacrylamide gel electrophoresis (Tris/borate/EDTA-PAGE) gel (Novex, San Diego, CA), which was then stained with ethidium bromide and photographed. The amplification product from an individual homozygous for the HindIII restriction site was subcloned directly into a PBluescript II-SK+ vector (Stratagene, La Jolla, CA) that had been digested with EcoRV and extended with a single 3' overhang. The resulting clone was sequenced and the polymorphic HindIII site identified.

**RESULTS**

Mapping the gene and characterization of the RFLP. Seven λ-phase clones were obtained from the first library screened. A normal human cosmid library was screened when it was determined the 5' end of the gene had not been initially cloned. Four phase and three cosmids clones spanning 70 kb were mapped by restriction analysis, including 25 kb upstream of the 40-kb gene. Exon positions were determined by comparing restriction sites from the sequenced regions of genomic subclones with the consensus genomic restriction map. The scheme in Fig 1 shows the p67phox genomic exon/intron map, an enlargement of the 5' end of the gene, and the fragments detected by Southern probing and genomic amplification. Only the HindIII sites are shown for clarity (complete restriction map available on request). Exon/intron boundary sequences for each of the 16 exons are shown in Table 1. The cDNA probe detects a 4-kb band at the 5' end of the gene in the absence of the allelic HindIII site, whereas only a single 2.3-kb band is detected in its presence because the downstream 1.7-kb fragment generated is devoid of exonic sequence. The next exonic segment lies in the 12-kb HindIII fragment further downstream. The HindIII RFLP site was localized to intron 2 by restriction analysis and sequencing. Amplification primers surrounding the RFLP site yielded a fragment that is cleaved by HindIII when the site is present. Allele 2 contains the recognition site for HindIII at base 201 of the 414-bp amplified fragment. The polymorphic restriction site has an estimated information content of 0.32 in the general population and follows a simple Mendelian pattern of inheritance.

Pedigree and RFLP-based diagnosis. Figure 2 shows a pedigree and Southern blot for the family affected by p67phox-deficient CGD, which shows a typical autosomal recessive inheritance pattern. The nitroblue tetrazolium dye (NBT) reduction test is normal in the parents but abnormal in the proband (who was shown to lack p67phox protein by immunoblot of her neutrophil cytosol) and was presumably abnormal in the brother who died in infancy. The parents (A and B) are obligate heterozygotes for a mutation responsible for p67phox deficiency, a CGD gene (single black shading); whether the mutation is the same in both parents is not known. The proband daughter (D) carries a mutation on both chromosomes (double black shading). Whereas the other living children are phenotypically normal, their carrier status is unknown (dashed outlines of one chromosome). The allele designation below each individual's symbol reflects the RFLP pattern in the Southern blot. The
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Fig 1. Physical map of p67\textsuperscript{Phox}. (A) The p67\textsuperscript{Phox} gene map on chromosome 1, with exons boxed and numbered and HindIII (H) restriction sites indicated as tick marks. Exon boxes are open within the coding region. A 5,000-bp scale is on the right. (B) Enlarged view of the genomic map. The allelic HindIII site lies in intron 2, just downstream of exon 2. Amplification primer sites, indicated by arrows, flank the allelic HindIII site. The PCR fragment containing the polymorphism is shown below. (C) HindIII fragments aligned with the genomic sequences that represent allelic fragments 1 and 2. A 500-bp scale is on the right. The sequence of the allelic HindIII site is shown below.

The proband (D) is homozygous for the 4-kb HindIII restriction fragment, designated as allele 1, as is the mother. The father is heterozygous with respect to the HindIII digest pattern, having both a 4-kb fragment (allele 1) associated with the CGD gene, and a 2.3-kb fragment (allele 2) associated with a normal p67\textsuperscript{Phox} gene. The fetus (C) and the two older brothers tested (E and F) are also heterozygous.

**Diagnosis by gene amplification.** When the DNA from each individual is amplified using the primer set flanking the normal allelic RFLP site, and the product digested with HindIII, the fragments obtained (Fig 3) were consistent with those predicted from the restriction pattern analysis of total genomic DNA shown in Fig 1. Amplified fragments derived from allele 1 have no HindIII restriction site, are not digested, and remain as a single fragment at 414 bp. The mother (B) and proband daughter (D) are homozygous for allele 1, thus their amplified DNAs appear as one band when treated with HindIII. Allele 2 derived fragments are digested with HindIII near the midpoint, and two bands at 196 bp and 218 bp are generated. The father (A), the two sons (E and F), and the fetus (C) are heterozygous for this restriction site. Therefore, three bands (414 bp, 218 bp, and 196 bp) are seen after HindIII digestion of the amplified products. As shown by the analysis based on this marker, the fetus and both normal sons have inherited at least one normal gene, which came from the father. The genomic 4-kb HindIII subclone (G) represents allele 1 and generates a PCR product that is not digested internally with HindIII.

**DISCUSSION**

Before the genes for the oxidase components missing or defective in CGD were cloned and characterized, the only methods available for prenatal diagnosis depended on the isolation of differentiated myeloid fetal cells. Newburger et al\textsuperscript{18} first used the NBT reduction test to diagnose CGD from an analysis of fetal granulocytes from placental vessel blood. This method has the benefit of being applicable to all types of CGD, but it is hampered by the difficulty of the sampling method, relative danger to the fetus, and the unreliability of a negative NBT test. More recently, in the case of X-linked disease, immunostaining for the cytochrome b\textsubscript{58} was used to detect chorionic villous macrophages sampled at 7 to 10 weeks of gestation.\textsuperscript{23} Whereas a positive result may be helpful in ruling out CGD, a negative result could again be an unreliable indicator of disease.

Accurate prediction of the fetal phenotype requires definition of the fetal genotype with respect to the relevant oxidase gene mutation. The most certain way to diagnose fetal cell DNA would be to find the mutation affecting the gene causing CGD in the particular family being studied. However, within each CGD subtype mutations causing disease have been found to be quite heterogeneous.\textsuperscript{24} No mutations have yet been reported for p67\textsuperscript{Phox}-deficient patients. With the chromosomal localization of the gene for the large subunit of cytochrome b\textsubscript{58} at Xp21, and its subsequent cloning,\textsuperscript{24} it became possible to perform a linkage analysis for prenatal diagnosis on DNA from high-risk families with the X-linked form of CGD.\textsuperscript{3,11,12} Knowledge of the correct linear order of linked markers is necessary to determine whether crossover has occurred in the chromosomes inherited by the fetus because of the large genomic distances involved in linkage analysis. There are two intragenic \textit{Nsi} I RFLPs\textsuperscript{10,33} and a microsatellite (GT repeat)\textsuperscript{34} that can be used for prenatal diagnosis in X-linked disease. The intragenic location of the p67\textsuperscript{Phox} gene HindIII RFLP minimizes the possibility of a recombination event that confounds analysis of inheritance patterns. The estimated recombination fraction for a random meiotic crossover event occurring between the intragenic marker and the mutation is less than 0.0004 (ie, <0.04% chance of recombination) for the p67\textsuperscript{Phox} gene, which is about 40 kb in size. Apart from the RFLP in the p67\textsuperscript{Phox} gene used in this study,\textsuperscript{14} there are no RFLPs reported for the other autosomal forms of CGD.

RFLP analysis of fetal DNA obtained by amniocentesis does not require knowledge of the mutation, but does require the family pedigree be informative for the relevant gene marker, as it is here. Both copies of the p67\textsuperscript{Phox} gene in the proband must contain a CGD mutation because this is a
recessive disorder. However, RFLP analysis in the mother does not distinguish the normal p67\textsuperscript{phox} allele from the CGD allele. In the absence of an intragenic crossover event, both the father and mother must have allele 1 p67\textsuperscript{phox} genes containing mutations that cause CGD. The normal brothers have inherited allele 2 from the father and are phenotypically normal. Thus, the RFLP is an informative marker in this family because the normal phenotype is predicted by the polymorphic marker designated allele 2. The fetus is shown to have inherited allele 2 from the father, and was expected to be phenotypically normal as well. This was confirmed at birth by phorbol myristate acetate-stimulated NBT test and chemiluminescence on neutrophils from cord blood (data not shown). Had the fetal DNA been homozygous for RFLP allele 1, it would have been predicted to be at least a carrier, and possibly affected by CGD. In that case umbilical-cord blood sampling and NBT testing, or a more extensive linkage analysis, would have been necessary to determine whether the fetus had CGD.

We have characterized the exonic organization of the p67\textsuperscript{phox} gene and described two methods for detecting the fetal inheritance pattern using this genetic marker. The first method depends on culturing the amniotic cells for 2 to 3 weeks to obtain sufficient DNA for direct restriction analysis. In the present study, the resulting Southern blot clearly indicates the presence of a normal allele in the fetus. The fetus went to term without further invasive testing, and was proven to be phenotypically normal. The second method, which required detailed knowledge of the genomic organization and precise mapping of the RFLP, involves diagnosis by gene amplification. Once a family is typed as to their allele status, gene amplification is applicable to chorionic villous sampling as early as 8 weeks’ gestation because of the small amounts of tissue required.

The RFLP analysis reported here adds a closely linked

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**Table 1. Exon/Intron Boundary Sequence for the p67\textsuperscript{phox} Gene**

<table>
<thead>
<tr>
<th>Exon number</th>
<th>Sequence</th>
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| 1           | gacagtagCTAGTCTTT  
             | 1TGGCTGAAAGttgggtttc  
             | 30  
             | TGGCTGAAAGttgggtttc  
             | 175  
             | TGGCTGAAAGttgggtttc  
             | 368  
             | TGGCTGAAAGttgggtttc  
             | 609  
             | TGGCTGAAAGttgggtttc  
             | 699  
| 2           | gacagtagCTAGTCTTT  
             | 2TGGCTGAAAGttgggtttc  
             | 30  
             | TGGCTGAAAGttgggtttc  
             | 175  
             | TGGCTGAAAGttgggtttc  
             | 368  
             | TGGCTGAAAGttgggtttc  
             | 609  
             | TGGCTGAAAGttgggtttc  
             | 699  
| 3           | gacagtagCTAGTCTTT  
             | 3TGGCTGAAAGttgggtttc  
             | 30  
             | TGGCTGAAAGttgggtttc  
             | 175  
             | TGGCTGAAAGttgggtttc  
             | 368  
             | TGGCTGAAAGttgggtttc  
             | 609  
             | TGGCTGAAAGttgggtttc  
             | 699  
| 4           | gacagtagCTAGTCTTT  
             | 4TGGCTGAAAGttgggtttc  
             | 30  
             | TGGCTGAAAGttgggtttc  
             | 175  
             | TGGCTGAAAGttgggtttc  
             | 368  
             | TGGCTGAAAGttgggtttc  
             | 609  
             | TGGCTGAAAGttgggtttc  
             | 699  
| 5           | gacagtagCTAGTCTTT  
             | 5TGGCTGAAAGttgggtttc  
             | 30  
             | TGGCTGAAAGttgggtttc  
             | 175  
             | TGGCTGAAAGttgggtttc  
             | 368  
             | TGGCTGAAAGttgggtttc  
             | 609  
             | TGGCTGAAAGttgggtttc  
             | 699  
| 6           | gacagtagCTAGTCTTT  
             | 6TGGCTGAAAGttgggtttc  
             | 30  
             | TGGCTGAAAGttgggtttc  
             | 175  
             | TGGCTGAAAGttgggtttc  
             | 368  
             | TGGCTGAAAGttgggtttc  
             | 609  
             | TGGCTGAAAGttgggtttc  
             | 699  
| 7           | gacagtagCTAGTCTTT  
             | 7TGGCTGAAAGttgggtttc  
             | 30  
             | TGGCTGAAAGttgggtttc  
             | 175  
             | TGGCTGAAAGttgggtttc  
             | 368  
             | TGGCTGAAAGttgggtttc  
             | 609  
             | TGGCTGAAAGttgggtttc  
             | 699  
| 8           | gacagtagCTAGTCTTT  
             | 8TGGCTGAAAGttgggtttc  
             | 30  
             | TGGCTGAAAGttgggtttc  
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             | TGGCTGAAAGttgggtttc  
             | 368  
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             | 609  
             | TGGCTGAAAGttgggtttc  
             | 699  
| 9           | gacagtagCTAGTCTTT  
             | 9TGGCTGAAAGttgggtttc  
             | 30  
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             | 368  
             | TGGCTGAAAGttgggtttc  
             | 609  
             | TGGCTGAAAGttgggtttc  
             | 699  
| 10          | gacagtagCTAGTCTTT  
             | 10TGGCTGAAAGttgggtttc  
             | 30  
             | TGGCTGAAAGttgggtttc  
             | 175  
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             | 368  
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             | 609  
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| 11          | gacagtagCTAGTCTTT  
             | 11TGGCTGAAAGttgggtttc  
             | 30  
             | TGGCTGAAAGttgggtttc  
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             | 368  
             | TGGCTGAAAGttgggtttc  
             | 609  
             | TGGCTGAAAGttgggtttc  
             | 699  
| 12          | gacagtagCTAGTCTTT  
             | 12TGGCTGAAAGttgggtttc  
             | 30  
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| 13          | gacagtagCTAGTCTTT  
             | 13TGGCTGAAAGttgggtttc  
             | 30  
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             | 175  
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             | 368  
             | TGGCTGAAAGttgggtttc  
             | 609  
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             | 699  
| 14          | gacagtagCTAGTCTTT  
             | 14TGGCTGAAAGttgggtttc  
             | 30  
             | TGGCTGAAAGttgggtttc  
             | 175  
             | TGGCTGAAAGttgggtttc  
             | 368  
             | TGGCTGAAAGttgggtttc  
             | 609  
             | TGGCTGAAAGttgggtttc  
             | 699  
| 15          | gacagtagCTAGTCTTT  
             | 15TGGCTGAAAGttgggtttc  
             | 30  
             | TGGCTGAAAGttgggtttc  
             | 175  
             | TGGCTGAAAGttgggtttc  
             | 368  
             | TGGCTGAAAGttgggtttc  
             | 609  
             | TGGCTGAAAGttgggtttc  
             | 699  
| 16          | gacagtagCTAGTCTTT  
             | 16TGGCTGAAAGttgggtttc  
             | 30  
             | TGGCTGAAAGttgggtttc  
             | 175  
             | TGGCTGAAAGttgggtttc  
             | 368  
             | TGGCTGAAAGttgggtttc  
             | 609  
             | TGGCTGAAAGttgggtttc  
             | 699  

Exon number is the same as in Fig. 1. Numbers above each exon line refer to the cDNA numbering beginning with the start of translation. The exonic sequence is in upper case and the intervening sequence is in lower case.
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Fig 3. Rapid genotype diagnosis of fetal DNA obtained by amniocentesis. PCR amplified products from genomic DNA were digested with HindIII, size fractionated by gel electrophoresis, and shown by staining with ethidium bromide. The lane designations A through F refer to family members defined in Fig 1. PCR products derived from cloned p67<sub>PHOX</sub> genomic DNA, and a control reaction in the absence of target DNA, are shown in lanes G and H, respectively. The positions of the allelic fragments are designated in the right margin. OX174RF/HaeIII digest molecular weight markers in base pairs flank lanes A through H.

and physically mapped amplifiable marker that may facilitate genetic studies in other pedigrees with mutations near p67<sub>PHOX</sub>. These data will stimulate the search for additional intragenic RFLPs in all four types of CGD, making prenatal diagnosis available to all families affected by this disorder.

ACKNOWLEDGMENT

The authors thank Dr John Gallin for his care of the patient and helpful discussions, Amy Muir for assistance with the initial library characterization, Dr Barbara Crandall for preparing the amniocentesis fetal cell culture, and Dr Phil Murphy for careful review of the manuscript. Sequenced regions of p67<sub>PHOX</sub> are available in Genbank, accession numbers U00776, U00777, U00778, U00779, U00780, U00781, U00782, U00783, U00784, U00785, U00786, U00787, and U00788.

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