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Cytochrome b_{558}-negative, Autosomal Recessive Chronic Granulomatous Disease: Two New Mutations in the Cytochrome b_{558} Light Chain of the NADPH Oxidase (p22-*phox*)

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Summary

Chronic granulomatous disease (CGD) is characterized by the failure of activated phagocytes to generate superoxide. Defects in at least four different genes lead to CGD. Patients with the X-linked form of CGD have mutations in the gene for the beta-subunit of cytochrome b_{558} (gp91-*phox*). Patients with a rare autosomal recessive form of CGD have mutations in the gene for the alpha-subunit of this cytochrome (p22-*phox*). Usually, this leads to the absence of cytochrome b_{558} in the phagocytes ($A22^0$ CGD). We studied the molecular defect in five European patients from three unrelated families with this type of CGD. P22-*phox* mRNA was reverse-transcribed, and the coding region was amplified by PCR in one fragment and sequenced. Three patients from one family, with parents that were first cousins, were homozygous for a single base substitution (G-297→A) resulting in a nonconservative amino acid change (Arg-90→Gln). This mutation was previously found in a compound heterozygote $A22^0$ CGD patient. Another patient, also from first-cousin parents, was homozygous for an A-309→G mutation in the open reading frame that predicts a nonconservative amino acid replacement (His-94→Arg). The fifth patient was also born from a first-cousin marriage and was shown to be homozygous for the absence of exon 4 from the cDNA. In this patient, a G→A substitution was found at position 1 of intron 4 in the genomic DNA. Therefore, the absence of exon 4 in the cDNA of this patient is due to a splicing error. Two additional polymorphisms were also identified—one silent mutation in the open reading frame (G-508←→A) and one A-640←→G mutation in the 3′ untranslated region of the p22-*phox* mRNA. This last mutation destroys a *Dra*III recognition site and is therefore potentially useful for RFLP analysis of CGD families.

Introduction

Chronic granulomatous disease (CGD) is a rare syndrome clinically characterized by recurrent, life-threatening pyogenic infections, by bacteria and fungi, of subcutaneous tissues, upper airways, lungs, bones, spleen, liver, and lymph nodes (Tauber et al. 1983). This disease is caused by the inability of the patients' phagocytes (neutrophilic granulocytes, eosinophilic granulocytes, monocytes, and macrophages) to generate superoxide, an essential precursor of reactive oxygen metabolites for the killing of various microorganisms. In normal phagocytes, NADPH:O_2 oxidoreductase (NADPH oxidase) reduces molecular oxygen to superoxide with electrons derived from NADPH. This enzyme is composed of several subunits, some of which are localized in the plasma membrane and others of which are localized in the cytosol (Smith and Curnutte 1991). During phagocytosis of microorganisms, the cytosolic components translocate to the membrane and integrate with the membrane-bound components into an enzymatically active complex. Defects in any one of four NADPH oxidase subunits cause

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inactivity of the enzyme, thus leading to CGD (Clark et al. 1989).

X-linked CGD is caused by mutations in the beta-subunit of cytochrome b_{558} (gp91-*phox*) (Royer-Pokora et al. 1986; Bolscher et al. 1991), whereas mutations in the alpha-subunit of this cytochrome (p22-*phox*) cause an autosomal recessive type of CGD (Dinauer et al. 1990). Together, gp91-*phox* and p22-*phox* form a heme protein with a very low midpoint potential, supposed to act as the terminal electron donor to oxygen (Segal 1989). Both cytochrome b_{558} subunits are membrane bound, and, for stable expression, require each other's presence (Parkos et al. 1989; Verhoeven et al. 1989). Both subunits have been cloned and characterized at the protein, cDNA, and genomic levels (Royer-Pokora et al. 1986; Dinauer et al. 1987; Teahan et al. 1987; Parkos et al. 1988; Orkin 1989; Dinauer et al. 1990). P22-*phox* is encoded by the CYBA gene on chromosome 16q24 (Dinauer et al. 1990), and gp91-*phox* is encoded by the CYBB gene on chromosome Xp21.1 (Royer-Pokora et al. 1986). The other forms of CGD are caused by deficiencies of two cytosolic NADPH oxidase components, a 47-kD phosphoprotein and a 67-kD protein (Clark et al. 1989). These last two forms of CGD are also transmitted in an autosomal way.

According to agreement among international investigators, CGD should be classified as follows: Deficiencies in the alpha-subunit of cytochrome b_{558} (p22-*phox*) lead to autosomal (A) CGD, designated as A22 CGD. When no p22-*phox* protein is detectable, the superscript 0 is added; with diminished protein levels, the superscript − is added; and, with normal protein levels, the superscript + is added (i.e., $A22^0$, $A22^-$, and $A22^+$). Similarly, deficiencies in the beta-subunit of cytochrome b_{558} (gp91-*phox*) lead to X-linked CGD, designated as $X91^0$, $X91^-$, or $X91^+$. Deficiencies in the cytosolic NADPH oxidase component p47-*phox* lead to $A47^0$, $A47^-$, or $A47^+$ CGD, and deficiencies in the cytosolic component p67-*phox* lead to $A67^0$, $A67^-$, or $A67^+$ CGD.

Until now, only four mutations in p22-*phox* have been identified (Dinauer et al. 1990, 1991), because of the fact that the resulting type of CGD is very rare (less than $1:5 \times 10^6$). With one exception, these mutations lead to loss of cytochrome b_{558} in the patients' phagocytes ($A22^0$ subtype of CGD). We now describe five additional patients from three different families with the $A22^0$ subtype of CGD. Moreover, two additional polymorphisms in the p22-*phox* mRNA are described.

Material and Methods

Classification of CGD Patients

Patients were diagnosed as CGD by lack of superoxide production after stimulation of purified neutrophils by phorbol myristate acetate or serum-treated zymosan. Assays used were chemiluminescence with lucigenin, oxygen consumption, and/or NBT slide test (Weening et al. 1985). The patients described in the present study were classified as $A22^0$ CGD by the following criteria: First, in the optical spectrum of the neutrophils, the heme peaks at 558, 529, and 427 nm were absent (Weening et al. 1985). Second, in the cell-free activation system, the defect was located in the membrane fraction of the neutrophils (Curnutte et al. 1987). Third, on western blots, both the p22-*phox* and gp91-*phox* bands were missing (Verhoeven et al. 1989). Fourth, the NBT slide tests of the parents were normal (Weening et al. 1985). In principle, these results can also be explained with gp91-*phox*-deficient patients (X-linked) in which the mother is extremely lyonized toward the normal phenotype or in which a new mutation has occurred in the germ line of one of the parents. For family S this was excluded by monocyte fusion, leading to NADPH oxidase complementation both with $X91^0$ CGD monocytes and with $A47^0$ CGD monocytes (Weening et al. 1985). For the other two families, this was not formally excluded, but the results described in the present study prove that patient 4 from family G and patient 5 from family dS are also $A22^0$ patients.

Preparation of RNA and DNA

Mononuclear leukocytes were purified from 20–100 ml of citrated blood by isopycnic centrifugation for 20 min at 1,000 *g* at 20°C on an isotonic Percoll suspension (Pharmacia Fine Chemicals, Uppsala) with a specific gravity of 1.076 g/ml. The cell layer on top of the Percoll suspension was collected and washed twice with PBS. The number of monocytes was calculated from a size-distribution pattern (Coulter counter). RNA was isolated by dissolving the mononuclear cells in 4 M guanidine thiocyanate and by centrifugation through 5.7 M cesium chloride (Chirgwin et al. 1979). Genomic DNA was isolated from circulating blood leukocytes (Sambrook et al. 1989).

Northern Blot Analysis

RNA corresponding to 10^7 monocytes was submitted to electrophoresis in 1.2% (w/v) agarose gels in the presence of formaldehyde and was blotted onto

GeneScreen Plus membrane filters (Sambrook et al. 1989). Blots were hybridized with the *Eco*RI/*Hin*cII fragment of p22-*phox* cDNA or with the H64 fragment of gp91-*phox* cDNA (Verhoeven et al. 1989). Probes were labeled by random priming (Sambrook et al. 1989). Hybridization with a probe for α-actin was used as a control for the quantification and integrity of the RNA.

Amplification and Sequencing of DNA

For analysis of mRNA sequences, first-strand cDNA was synthesized from mononuclear-cell RNA and was amplified by PCR under conditions described by Bolscher et al. (1991), with some modifications. The RNA strand was cleaved from the RNA:DNA hybrids by 30 min of incubation with 1 Unit of RNAse H (Boehringer-Mannheim, Germany). To improve the efficiency of amplification of sequences high in G + C content, the reaction mix contained 175 μM dGTP and 25 μM 7-deaza-2′-dGTP; remaining dNTPs were each at 200 μM. The entire p22-*phox* cDNA coding region was amplified with the use of primers 1 and 2 (table 1). The PCR products were cloned in M13 *mp*18/*mp*19 and were sequenced with the dideoxynucleotide chain termination method using the Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland). Because of the possibility of heterozygosity, 10 independent clones per patient were sequenced.

The mutations were identified in genomic DNA as follows: Exon 4 plus boundary sequences were amplified with PCR using primers 3 and 4 (table 1) corresponding to sequences on the flanking introns. Conditions of the PCR were as described above but without 7-deaza-2′-dGTP. The PCR product was purified with the Geneclean II kit (BIO 101, Inc., La Jolla, CA) to remove the primers and nucleotides. Two hundred nanograms of the purified DNA samples were annealed with 40 ng of one of the primers used for amplification, by first being heated for 3 min at 100°C and then being chilled on ice water in the presence of 10% dimethylsulfoxide. Direct sequence analysis was performed with the Sequenase version 2.0 kit.

Allele-specific Hybridization

Allele-specific hybridization was performed essentially according to the method described by Bolscher et al. (1991) to identify single base substitutions in exon 4 and in the exon 4/intron 4 boundary sequence. For this purpose, exon 4 plus intron boundary sequences were amplified from genomic DNA as described above. Sense and antisense allele-specific oligonucleotide (ASO) probes were prepared for a CGG→CAG substitution, at nucleotide position 297 (numbered with respect to the published cDNA sequence [Parkos et al. 1988]), that predicts an Arg-90→Gln substitution (Arg ASO = probe 6 [table 1], and Gln ASO = probe 7 [table 1]); for a CAT→CGT substitution at nucleotide position 309 that predicts a

Table 1

PCR Primers and ASO Probes Used in This Study

Primer or Probe	Sequence[a]
Primer 1 (NT2)	5′-tactggatccAGTGTCCCAGCCGGGTTCGTGTCGCCAT-3′
Primer 2[b] (NT673)	5′-tagtaagcttCGCTGCGTTTATTGCAGGTGGGT-3′
Primer 3 (intron 3)	5′-tactggatccTTGTGGGTAAACCAAGGC-3′
Primer 4[b] (intron 4)	5′-tagtaagcttTGGAAAAACACTGAGGTAAGTGGGGGT-3′
Primer 5 (NT428)	5′-tactggatccAGTGGACGCCCATCGAGCCCAAG-3′
Probe 6 (Arg ASO)	5′-ACTATGTTCGGGCCGTCCTG-3′
Probe 7[b] (Gln ASO)	5′-CAGGACGGCCTGAACATAGT-3′
Probe 8 (His ASO)	5′-CCGTCCTGCATCTCCTGC-3′
Probe 9[b] (Arg ASO)	5′-GCAGGAGACGCAGGACGG-3′
Probe 10 (GTGA ASO)	5′-GCATCTCCTGTGAGTCCC-3′
Probe 11[b] (ATGA ASO)	5′-GGGACTCATAGGAGATGC-3′
Probe 12 (ASO CCG)	5′-GCGGCCCCCGGCCGAGGCCC-3′
Probe 13[b] (ASO CCA)	5′-GGCCTCGGCTGGGGGCCG-3′
Probe 14 (ASO A-640)	5′-CTGCCCTCCCACCAGGTGCAC-3′
Probe 15[b] (ASO G-640)	5′-GTGCACCTGGCGGGAGGGCAG-3′

[a] Lowercase letters indicate the *Bam*HI and *Hin*dIII restriction sites for sense and antisense, respectively.

[b] Antisense.

His-94→Arg substitution (His ASO = probe 8 [table 1], and Arg ASO = probe 9 [table 1]); and for an intron 4 donor splice site GTGA→ATGA substitution (normal ASO = probe 10 [table 1], and mutated ASO = probe 11 [table 1]). In addition, ASO probes were also prepared for two polymorphisms: (1) G-508←→A, which changes the Pro-160 codon CCG into the alternative Pro codon CCA (ASO CCG = probe 12 [table 1], and ASO CCA = probe 13 [table 1]) and (2) A640←→G in the untranslated 3′ region of the mRNA (ASO A-640 = probe 14 [table 1], and ASO G-640 = probe 15 [table 1]). For this purpose the exon 6 sequences from genomic DNA were amplified with primers 5 and 2 (table 1). The PCR conditions were the same as those used for exon 4 amplification.

Results

We investigated the genetic basis of cytochrome b_{558} deficiency in five CGD patients from three unrelated families. Family S has been described elsewhere (Corbeel et al. 1978; Weening et al. 1985). The parents are first cousins of Turkish origin; the mother suffered from severe chronic polyarthritis and died in 1986. The eldest child (a boy) died at the age 5 years from generalized salmonella infection. The next three children (patients 1 [female], 2 [male], and 3 [female]) were investigated. Patient 3 died in 1991 from generalized amyloidosis. The youngest child (a girl) is clinically well. Patient 4 (female) from family G (of Italian origin) and patient 5 (female) from family dS (of Portuguese origin) have not been reported before. Both marriages were consanguineous (first cousins); the parents are clinically well.

The mRNA of the patients' monocytes contained both gp91-*phox* mRNA and p22-*phox* mRNA, as detected on northern blot (not shown). In comparison to α-actin, apparently normal sizes and amounts were found.

The p22-*phox* mRNAs of the five patients were amplified in one fragment. Figure 1 shows the analysis of the PCR fragments on an agarose gel. The PCR fragment of patient 5 is apparently smaller than those of the other patients and of normal control cells.

Sequence analysis of the cDNA PCR fragments revealed a G-297→A transition in exon 4 in patients 1–3, predicting a nonconservative Arg-90→Gln substitution. This mutation was found in all 30 clones examined from these patients, rendering it most likely that the patients are homozygotes for this transversion. In patient 4, all 10 clones contained an A-309→G

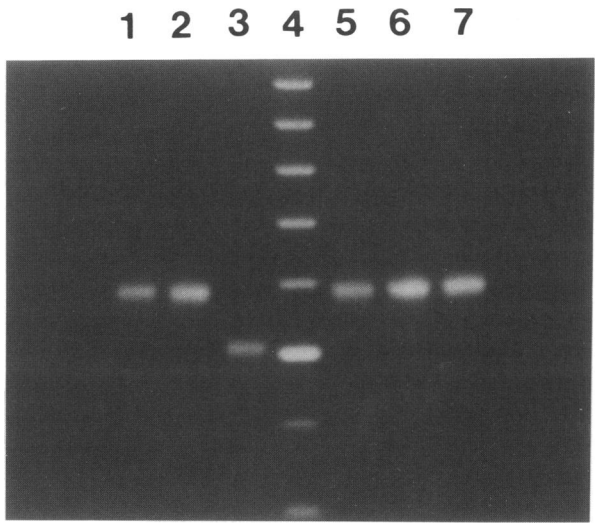

Figure 1 Size analysis of the PCR-amplified fragments of p22-*phox* cDNA. Ten percent of the PCR-amplified DNA products were run on a NuSieve 2:1 agarose gel and were stained with 0.05% (w/v) ethidium bromide. Lane 1, Control. Lane 2, Patient 4. Lane 3, Patient 5. Lane 4, Molecular-weight markers (1, 100, 1,000, 900, 800, 700, 600, 500, and 400 bp). Lane 5, Patient 1. Lane 6, Patient 2. Lane 7, Patient 3.

transition in exon 4, which predicts a homozygous nonconservative His-94→Arg substitution. In patient 5, the sequences of exon 4 were absent in all 10 clones, which explains the smaller size of the PCR fragment shown in figure 1. This was confirmed with direct sequencing (fig. 2).

To investigate the cause of these aberrations at the genomic level, we amplified exon 4 and the flanking intron sequences by PCR (see Material and Methods). This product was directly sequenced; the results confirmed those found with the cDNA PCR products for patients 1–4 (fig. 3). The mother of patient 4 and younger sister, mother, and father of patients 1–3 were found to be heterozygotes. With the genomic DNA from patient 5, a PCR product of the expected size was obtained, indicating that the absence of exon 4 in the p22-*phox* mRNA was not due to a gene deletion of this exon. Sequencing of the donor splice region revealed a single base substitution in the consensus donor splice sequence of G→A, at position 1 (fig. 2). Therefore, we conclude that the absence of exon 4 in the p22-*phox* mRNA of patient 5 is due to an mRNA splicing defect resulting in exon skipping. This in-frame deletion predicts a p22-*phox* polypeptide that is 28 amino acids shorter than normal. Figure 4 sum-

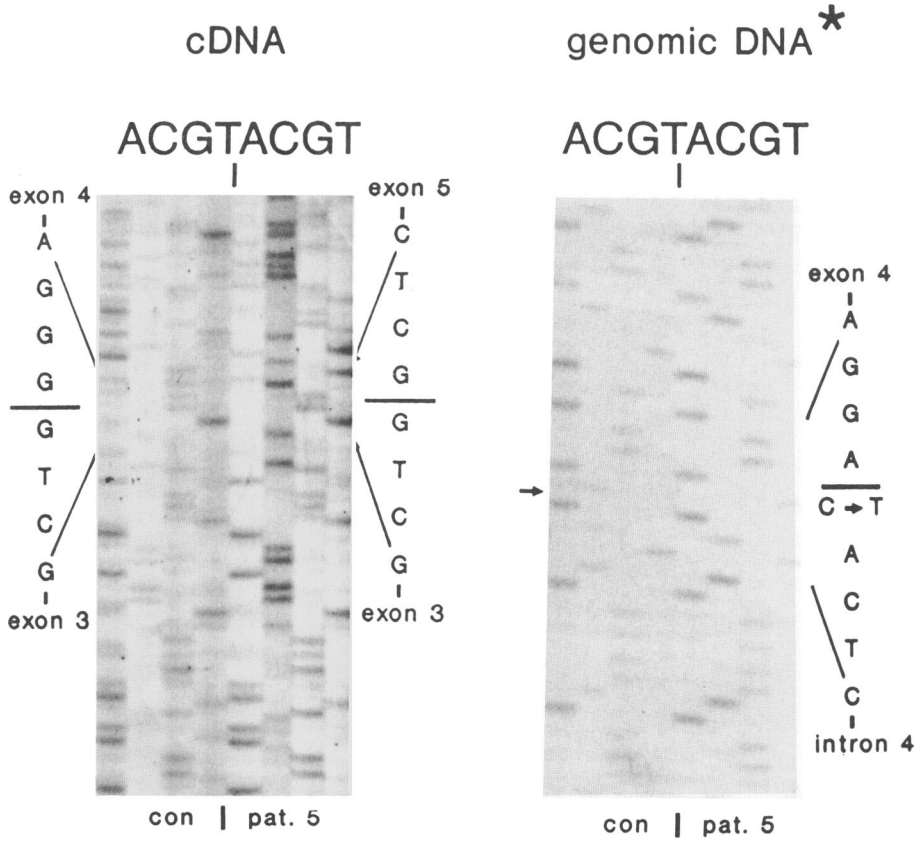

Figure 2 Sequence analysis of PCR-amplified p22-*phox* cDNA and genomic DNA of patient 5. The arrow points to the splice junction where exon 4 is skipped (left) and where the mutation in the exon 4/intron 4 boundary is located (right). Control and patient sequences are indicated. The asterisk (*) indicates the antisense sequence.

marizes the results of the mutation analyses in the three A22^0 CGD families.

The observed mutations were confirmed by ASO hybridization on PCR-amplified genomic DNA, with the probes specified in table 1. All probes containing the mutated sequence hybridized only with DNA from the patients in which the mutation was found. ASO hybridization with 78 alleles of unrelated donors was negative. The parents and sister of patients 1–3 and the mother of patient 4 reacted with both the normal and the mutant probes; other relatives were not available for study.

In the course of these studies we also detected two polymorphisms in the p22-*phox* mRNA: one (present in patients 1–3) located at nucleotide position G-508→A, which changes the Pro-160 codon CCG into the alternative Pro codon CCA; and one at nucleotide position A-640→G in the untranslated 3′ region of the mRNA. ASO hybridization demonstrated that both the Pro-160 codon CCG and the Pro-160 codon CCA are present in normal genomic DNA: of the 25 normal female donors tested, none were homozygous for G-508, 21 were homozygous for A-508, and 4 were heterozygous for both alleles. Also, A-640 and G-640 were found in normal genomic DNA: of the 40 normal female donors tested, 5 were homozygous for A-640, 9 were homozygous for G-640, and 26 were heterozygous for both alleles. C-639 through G-647 is a recognition site for *Dra*III when A-640 is included. Therefore, the A-640/G-640 polymorphism may be used for RFLP analysis of CGD families.

Discussion

The mutation found in p22-*phox* of patients 1–3 (family S.) confirms at the molecular level the genetic difference between this family and both the classical X-linked CGD (gp91–*phox* deficiency) and the com-

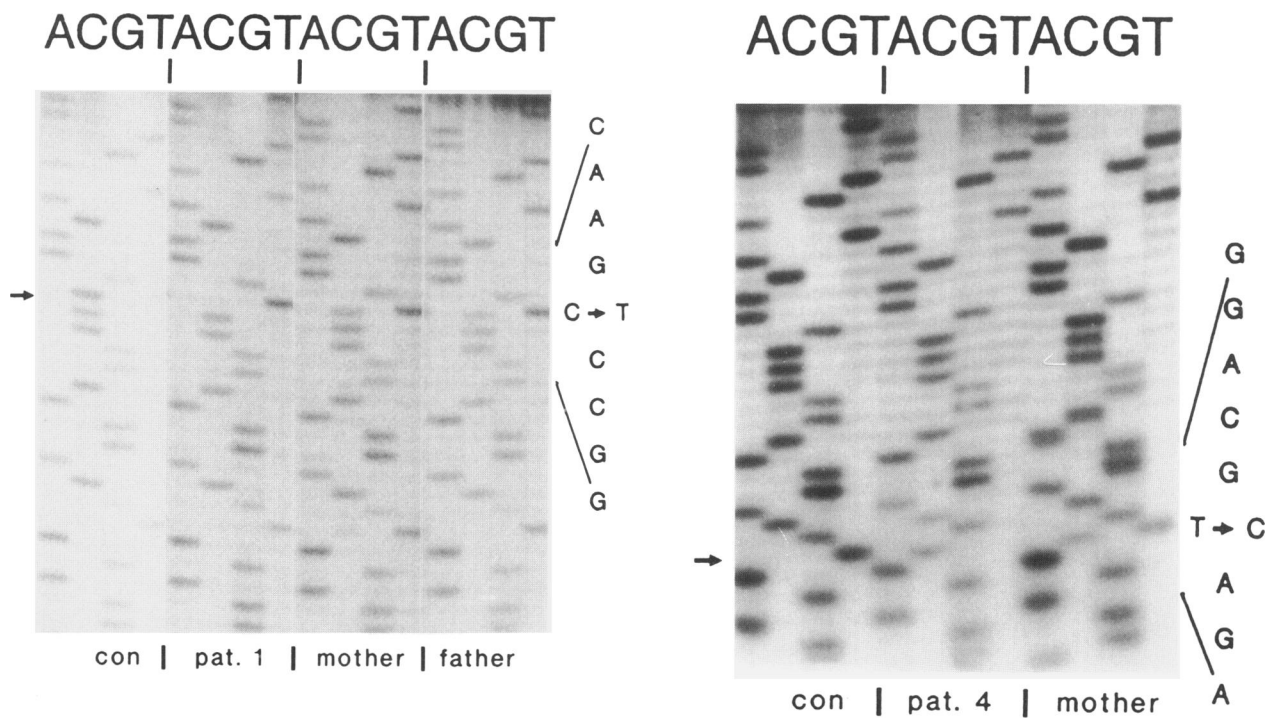

Figure 3 Sequence analysis of PCR-amplified p22-*phox* genomic DNA of patient 1 and her parents and of patient 4 and her mother. Arrows point to the mutations. Control and patient sequences are indicated. The parents show both the normal and the mutated sequence. The results obtained with the DNA from patients 2 and 3 were identical to those shown for patient 1. All strands were sequenced in the antisense direction.

mon autosomal type of CGD (p47-*phox* deficiency). In fact, the autosomal, cytochrome b_{558}–negative type of CGD was first described in this family (Weening et al. 1985). The mutation found (i.e., Arg-90→Gln) is identical to the mutation described by Dinauer et al. (1990) in a compound heterozygote Ab^0 CGD patient (table 2). Possibly, the Arg→Gln substitution induces a different conformation of the protein, thus decreas-

Table 2

Mutations in p22-*phox* in A22 CGD

Reference and Patient(s)	Sex	CGD Subtype	Mutation	p22-*phox* mRNA	p22-*phox* Protein and Heme	Nucleotide Change	Amino Acid Change[a]
Dinauer et al. 1990:							
L.N.	(female)	$A22^0$	>10 kb deletion (homozygous)	Absent	Absent	NA	NA
G.S.	(male).....	$A22^0$	1) Point deletion	Present	Absent	C-272 deletion	Frameshift
			2) Missense	Present	Absent	G-297→A	Arg-90→Gln
O.P.	(female)	$A22^0$	Missense (homozygous)	Present	Absent	C-382→A	Ser-118→Arg
Dinauer et al. 1991:							
I.L.	(female)	$A22^+$	Missense (homozygous)	Present	Normal	C-495→A	Pro-156→Gln
Present study:							
Family S.	(two females and one male)	$A22^0$	Missense (homozygous)	Present	Absent	G-297→A	Arg-90→Gln
A.G.	(female)	$A22^0$	Missense (homozygous)	Present	Absent	A-309→G	His-94→Arg
W.d.S.	(male).....	$A22^0$	Missense (homozygous)	Present	Absent	Intron 4 donor splice site G(+1)→A	NA

[a] NA = not applicable.

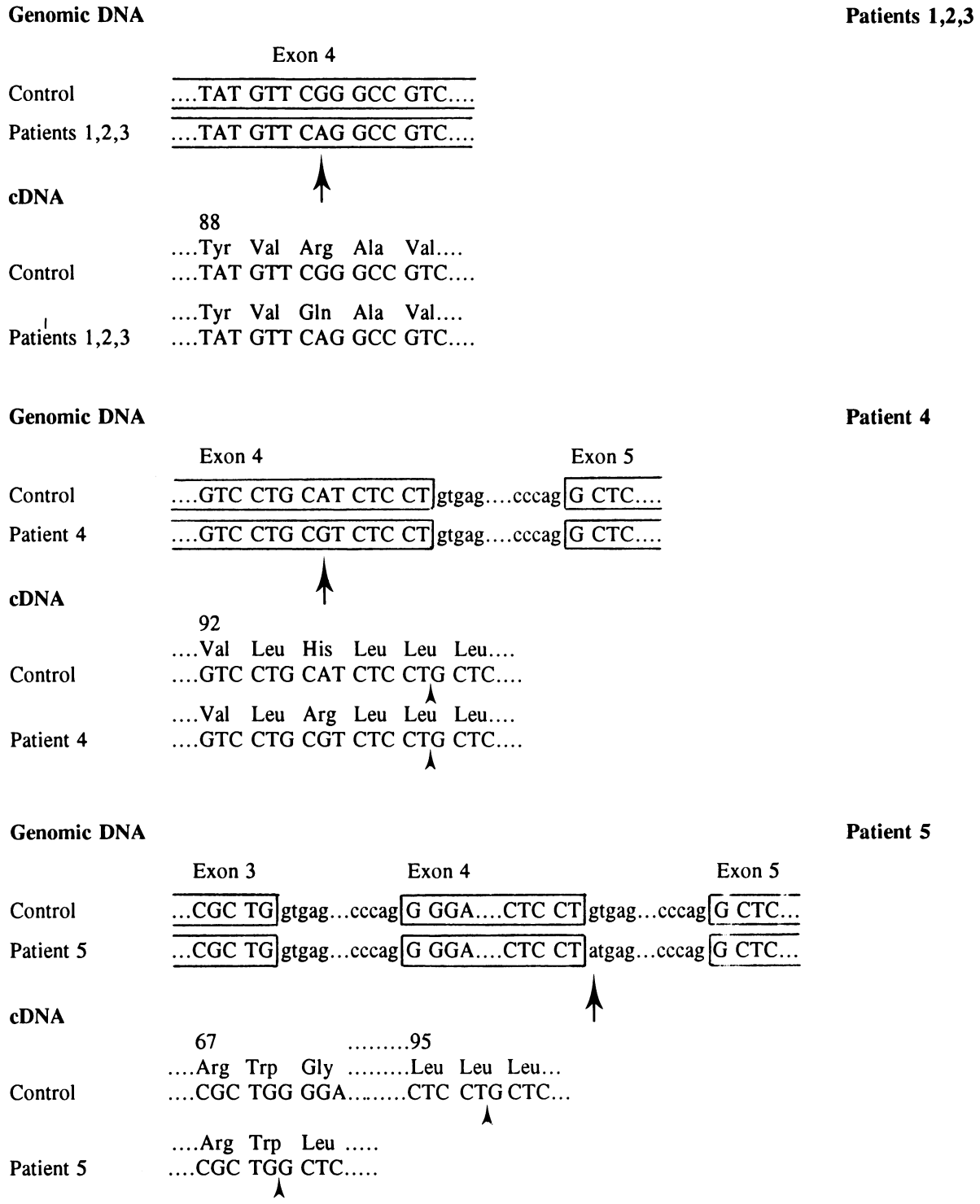

Figure 4 Mutations in the p22-*phox* gene that cause $A22^0$ CGD. Short stretches are shown of the nucleotide sequences, as are predicted amino acid sequences that contain the mutations. Uppercase letters indicate coding sequences, and lowercase letters indicate intron sequences. Exon sequences in genomic DNA are shown within boxes. Arrowheads in the cDNA sequences show the splice sites. Arrows indicate the mutations in the patients. The amino acid numbering on top of the sequences is according to the protocol of Parkos et al. (1988).

ing its stability and/or its association with the beta-chain of cytochrome b_{558}. The G-508↔A polymorphism found in this family was not reported by Dinauer et al. (1990). Thus, the Arg-90→Gln mutation that caused CGD in family S has probably arisen independently of the one described by Dinauer et al. (1990).

In patient 4, the His-94→Arg substitution in p22-*phox* removes the histidine that is probably involved in heme binding (Dinauer et al. 1990; Quinn et al. 1991). Although p22-*phox* contains two histidines, the His-72 is polymorphic and may be replaced by Tyr-72, without consequences for NADPH oxidase activity (Dinauer et al. 1990). Cytochrome b_{558} contains two heme groups per molecule, one of which is probably bound to two histidines in the beta-chain and the other of which is probably bound to one histidine each in the alpha chain and beta chain (Quinn et al. 1991). If this concept is right, substitution of His-94 in p22-*phox* necessarily results in loss of heme from cytochrome b_{558}—and thus in loss of NADPH oxidase activity. However, because the neutrophils of patient 4 did not contain measurable amounts of cytochrome b_{558} subunits on western blot either, the His-94 substitution affects the stability and/or the association of p22-*phox* with gp91-*phox* as well.

In patient 5, we found a G→A substitution at position 1 of intron 4 of the CYBA gene in the consensus donor splice site of intron 4. Apparently, this mutation leads to skipping of exon 4 during p22-*phox* mRNA splicing. This is the first known example of exon skipping as a cause of $A22^0$ CGD. Previously, several splice-site mutations have been found that lead to partial or total exon skipping in gp91-*phox* mRNA splicing, thus causing X-linked CGD (Schapiro et al. 1991; de Boer et al., in press; Roos et al., in press). In patient 5, exon 4 skipping leads to an in-frame deletion (fig. 4) and therefore to a polypeptide shortened by 28 amino acids. This too, leads to absence of both p22-*phox* protein and gp91-*phox* protein in the patients neutrophils.

Until now, mutations in p22-*phox* have been described in seven families with A22 CGD (table 2). In six of these families, the heme signal and the subunits of cytochrome b_{558} were undetectable ($A22^0$). In one patient (Dinauer et al. 1991) with a homozygous Pro 156→Gln substitution in p22-*phox*, cytochrome b_{558} heme and protein were still present, but NADPH oxidase activity was lost, presumably because of this mutation ($A22^+$). Thus, seven mutations were found in seven A22 CGD families, indicating that this type of CGD—like the X91 type (Bolscher et al. 1991; Roos et al., in press)—is very heterogeneous in nature. Until now, only four polymorphisms have been recognized in p22-*phox* (table 3), and none at all have been recognized in gp91-*phox*. Apparently, small changes in the structure of the cytochrome b_{558} subunits already lead to instability and/or loss of function of this protein.

Table 3

Polymorphisms in p22-*phox*

Reference and Nucleotide	Amino Acid[a]
Dinauer et al. 1990:	
C-243↔T	His-72 ↔Tyr
C-549↔T	Ala-174 ↔Val
Present study:	
G-508↔A	Pro 160↔Pro
A-640↔G	NA

[a] NA = not applicable (polymorphism in 3′ untranslated region).

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