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Molecular characterization of the genetic defect and functional reconstitution of the NADPH oxidase activity in B-lymphoblast from patients with X-linked Chronic Granulomatous Disease

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INTRODUCTION

This thesis reports the results of several molecular studies carried out on three patients affected by Chronic Granulomatous Disease (CGD). CGD is an uncommon inherited disorders in which phagocytic cells fail to produce antimicrobial oxidants. Affected individuals suffer from recurrent and often life threatening bacterial and fungal infections, and they usually die at very young age. Since current clinical therapies are poorly effective in controlling the disease, a therapeutic strategy based on gene transfer is potentially of great interest.

The experimental work present in this thesis has been divided into three main sections: the first one describes the present knowledge about CGD, while the other two concern the characterization of the genetic defect leading to the disease and the correction of the genetic defect in cells from affected patients respectively. Of particular interest is the functional reconstitution of oxidase activity which was achieved by gene transfer in lymphoblastoid cell lines derived from the patients. The correction of the defect was achieved by transduction of these cell lines with a retroviral vector carrying the normal cDNA of the gene which was found to be mutated in the patients.

The results presented in this thesis represent the first step in a gene therapy program aiming at the correction of the genetic defect by *ex vivo* gene transfer into the hematopoietic stem cells of the patients.

MOLECULAR GENETICS OF CHRONIC GRANULOMATOUS DISEASE

1. Clinical features

1.1 Definition

Chronic granulomatous disease (CGD) is a rare hematological disease with an estimated incidence between 1:250,000 and 1:500,000 (235). The disease is due to an heterogeneous group of genetic defects that are responsible for recurrent, severe bacterial and fungal infections usually involving the skin, soft tissues, respiratory tract, lymph nodes, liver and spleen (254), (14), (125), (138), (278), (92), (60). The disease can be inherited in either an X-linked or an autosomal recessive fashion. Infectious episodes can be fatal, and therapy requires prolonged prophylaxis with antibiotic in order to reduce and control infections. Phagocytes (neutrophils, monocytes and eosinophils) from affected individuals can ingest microorganisms but they are not able to destroy them after ingestion due to defects in the enzymatic complex producing antimicrobial agents with oxidative properties like superoxide anion. The process responsible for the production of these reactive chemical species is called respiratory burst. Superoxide anion is produced by a NADPH oxidase, a membrane bound enzymatic complex.

1.2 Clinical presentation

CGD should be considered in any individual with recurrent purulent infections caused by fungi or catalase-positive bacteria. Patients are represented mostly by children with a history of recurrent infections.

CGD is as a very heterogeneous syndrome. This is apparent in the type of infectious micro-organisms, in the different infected tissues, in the frequency of the infectious episodes and in the age at which patients present with the infections. The clinical course mirrors the heterogeneity in the molecular pathogenesis of the disease. In general, it has been described (303), (87), (171), that patients with defects in cytochrome b_{558} have a more severe clinical course than those with defects in cytosolic NADPH oxidase components (see below). There is no correlation between the amount of superoxide generation and the severity of the disease. Generally, patients die at very young age. However, same long surviver patients have been described (251).

The most common clinical findings are reassumed in table 1. Although any organs can be affected, the skin, the mononuclear phagocyte system (spleen, liver, and lymph nodes), the respiratory and gastrointestinal tracts are usually involved.

The most serious infections take place in spleen, liver, lymph nodes and lung, reflecting the accumulation of infecting microorganisms by phagocytic cells that are not able to kill them (125). Cervical and other lymph nodes can become enlarged in the course of the disease: a possible evolution is represented by spontaneous rupture and drainage. Hepatomegaly and splenomegaly are very often detectable. Liver involvement may progress to abscess formation requiring surgical intervention (125).

Skin infections, mainly represented by pyogenic dermatitis, furunculosis and subcutaneous abscesses (125), (278), (313), are very common and can be prominent in adults with otherwise mild disease (20).

Infections of the lower respirator tract are also common. Lobar, bronchial or diffuse and generalized pneumonia can occur with high frequency (14), (80), (315), (96). On the contrary lung abscesses are not so

TABLE 1

Clinical findings in 168 patients with Chronic Granulomatous Disease

Finding	*Number of patients involved*
Marked lymphadenopathy	137
Pneumonitis	134
Dermatitis	120
Hepatomegaly	114
Onset by age 1 year	109
Suppuration of nodes	104
Splenomegaly	95
Hepatic-perihepatic abscess	69
Osteomyelitis	54
Onset with dermatitis	42
Onset with lymphadenitis	38
Facial periorificial dermatitis	35
Persistent diarrhea	34
Septicemia or meningitis	29
Perianal abscess	28
Conjunctivitis	27
Death from pneumonitis	26
Persistent rhinitis	26
Ulcerative stomatitis	26

From: Johston R.B. JR, Newman S.L., 1977.

common. The consequences of repeated infectious episodes are granulomatous infiltration and fibrosis.

The oropharynx (132) and gastrointestinal tract (7) are frequent sites of recurrent infections. Ulcerative stomatitis and gingivitis can be found as well as esophagitis. The recurrent infections in the gastrointestinal tract lead to different syndromes mimicking pyloric stenosis, eosinophilic gastroenteritis or bowel disease. Another characteristic feature is the luminal narrowing of the gastric antrum with persistent vomiting (87). Rectal abscesses, perianal abscesses and fistulas are not uncommon (7).

Osteomyelitis has been found in about one third of patients, especially in metacarpals and metatarsal bones (125), (278), (314).

Urinary tract infections are less common. Only 7% of a group of 168 patients suffered from this type of infections (125). As well as in other apparati, obstructive syndromes can take place in the urinary tract (14), (278), (87), (63), (319), (145). Amyloidosis (14), glomerulonephritis (288), renal abscesses (86) and granulomatous inflammation of the kidney parenchyma have also been reported (28).

Disseminated infections with bacteriemia and/or meningitis can less frequently occur (17% of 168 cases described) (125), (85).

Destructive chorioretinitis (173), conjunctivitis (125), thyroiditis (102), pericarditis (125) and brain abscess (125) can also be found in patients affected by CGD.

1.3 Infecting organisms

The most frequent bacterial species associated with infection at almost any sites are *S.Aureus*, enteric bacteria and *Aspergillus*. In table 2 the infectious agents usually isolated from patients with CGD are listed. *S aureus* and enteric bacteria are the microorganisms most frequently

TABLE 2

Organism	Number of patients involved
Staphylococcus aureus	87
Klebsiella-Aerobacter organisms	29
Escherichia coli	26
Serratia marcescens	16
Pseudomonas organisms	15
Staphylococcus albus	13
Aspergillus organisms	13
Candida albicans	12
Salmonella organisms	10
Proteus organisms	9
Streptococci	9
Nocardia organisms	4
Mycobacteria	4
Paracolobactrum organisms	4
Actinomyces organisms	2
Other enteric bacteria	9

From: Johnston R.B. JR, Newman S.L., 1977.

cultured from spleen and liver samples (125), (278). The same organisms are commonly found also in lower respiratory tract infections. *Salmonella* is the most commonly isolated organism both from the blood and fatal infectious episodes (154).

Interestingly, encapsulated *Streptococcus* and *Hemophilus* species (which do not produce catalase), are very rarely found in CGD patients. They fall victim to the microbicidal effects of their endogenously produced H_2O_2 that is released into the phagocytic vacuole and converted to HOCl in the presence of myeloperoxidase or to ·OH in the presence of iron.

1.4 Pathologic findings

Specimens from acutely infected sites show a necrotic inflammatory process associated with suppuration. If the infection has been prolonged, granulomas are present, with multinucleated giant cells, macrophages, lymphocytes, and plasma cells (278). The formation of these granulomas appears to be secondary to the prolonged intracellular residence of microorganisms (124). The abundance of mononuclear phagocytes in the liver, spleen, lungs, and lymph nodes makes these organs particularly susceptible to the formation of granulomatous lesions. When multiplying organisms are released from one phagocyte, they are usually ingested by another one. This process recruits additional phagocytes with the eventual formation of granulomatous masses. These masses are the most typical lesion found in patients affected by CGD especially in the late phases of the disease. They can reach big dimension especially in lung and liver.

1.5 General laboratory findings

During infections, a neutrophilic leukocytosis is frequent and may be associated with an elevated erythrocyte sedimentation rate. Anemia appears to be secondary to chronic infections; resolution usually occurs during disease-free intervals. A polyclonal hypergammaglobulinemia is present, with elevated serum concentrations of IgG, IgM, and IgA (124), (14). Other tests of immune function are normal (14), with the rare exception of abnormal lymphocyte activation or chemotaxis, thought to be due to serum inhibitors (301), (49).

1.6 Diagnosis

In a patient with clinical symptoms suggestive of CGD, the diagnosis has to be confirmed by the impairment of the neutrophils superoxide production upon treatment with an appropriate stimulus (phorbol-myristate acetate, for example). Many tests are available for measuring neutrophil oxidase activity. It is possible to measure oxygen consumption (with an oxygen electrode), superoxide generation (reduction of ferri-cytochrome c) or production of cytochrome peroxide (oxidation of homovanillic acid)(304),(305), (238). Chemiluminescence with luminol or lucigenin is also often used to measure oxidase activity (303) as well as flow cytometric methods (233). However, the most common clinical test is the NBT (nitro blue tetrazolium) test. In this test the reduction of NBT to its purple formazan crystal by superoxide is tested. Neutrophils are incubated with the pale yellow dye NBT, activated (e.g. with phorbol-myristate acetate) and scored microscopically for deposits of black formazan (176). In the most common form of CGD, no NBT reduction is observed in any of the cells. In some variant forms, however,

a percentage of cells may contain small amounts of formazan, a finding indicative of a greatly diminished respiratory burst in neutrophils. NBT is also useful in detecting the carrier state in X-linked CGD families. In this case, approximately 50% (depending on the lyonization pattern) of neutrophils are able to reduce NBT.

Once the diagnosis of CGD has been determined, it is necessary to differentiate the four subgroups (see paragraph n. 5 for details). Each subgroup is characterized by a genetic defect in the gene encoding for one of the four main NADPH-oxidase components (p22-*phox*, gp91-*phox*, p47-*phox* and p67-*phox*). Only one subtype (gp91-*phox*) is characterized by X-linked transmission (X91), while the other ones (p22-*phox*, p47-*phox* and p67-*phox*) by autosomal transmission (A22, A47 and A67) respectively. The consequence of the defect is the absence or the reduction of the specific protein in the neutrophils of the patients. According to the degree of reduction, different phenotypes can be recognized: $X91^0/A22^0$ when no protein can be detected; $X91^-/22^-$ when subnormal amounts of proteins are present and $X91^+/A22^+$ when normal amounts of proteins are present. The defects in the p47-*phox* and p67-*phox* genes, so far described, lead to complete absence of the proteins ($A47^0$, $A67^0$).

Laboratory differentiation between the different subgroups, begins with western blot analysis of neutrophil lysates with antibodies against p22-*phox*, gp91-*phox*, p47-*phox* and p67-*phox*. In case of $A47^0$ or $A67^0$ CGD, the distinction is easy, since the lack of reactivity with the relevant antibodies is the rule. In case of A22 or X91 CGD the distinction can be more difficult, since both subunits of cytochrome b_{558} are absent in $A22^0$ as well as in $X91^0$ CGD (211), (291), and + and - variants are known to exist (73), (76), (251), (237), (159). When both subunits are undetectable, distinction between $A22^0$ and $X91^0$ CGD can be made by searching for carriers in the family of the patients by the NBT slide test. The presence of

neutrophils with functional and neutrophils with non-functional NADPH oxidase in obligate heterozygotes (e.g. the mothers of the patients) proves the X-linked nature of the disease, and thus points to a deficiency in gp91-*phox*. Of course, if the patient is female, this in itself is an indication that the disease probably has an autosomal origin, and hence may be caused by a deficiency in p22-*phox*. It must be kept in mind, however, that extreme lyonization in carriers of gp91-*phox* deficiency may lead to clinical problems as well(31), (62). When both subunits of cytochrome b_{558} are detectable on protein blots with the appropriate antibodies, a (relative) deficiency of NADPH oxidase activity of the patient's neutrophil membranes in the cell-free system will prove a defect in cytochrome b_{558}.

2. Normal microbicidal mechanisms of phagocytic cells

Phagocytic leucocytes (neutrophils, eosinophils, monocytes and macrophages) ingest and retain microorganisms in intracellular vacuoles (phagosomes), where they are exposed to cell-generated antimicrobial factors and killed. Neutrophils use several methods to destroy invading microbes. These methods can be classified according to their dependence on chemical molecular oxygen.

2.1 Oxygen-independent mechanisms

Several lines of evidence support a role for microbicidal activity that occurs in the absence of oxygen. First, the efficacy of oxygen-independent mechanisms can be demonstrated by the bactericidal activity of neutrophils in oxygen-depleted systems (169), (289). Secondly, neutrophils from patients with chronic granulomatous disease, which are unable to generate microbicidal oxygen metabolites, can kill at least some of an inoculum of most bacteria (306). Thirdly, constituents of neutrophil granules have bactericidal capacity.

Studies of fractions from disrupted neutrophils have localized the antibacterial protein activity to cytoplasmic granules (270), (271). Several antibacterials protein have been described, a few of which merit mention. Table 3 describes the best known proteins indicating subcellular location, optimal pH activity and susceptible microorganisms.

TABLE 3

Bactericidial protein	*Subcellular location*	*Optimal pH*	*Susceptible species*
Cationic antimicrobial proteins (Mr=37,000 and 57,000)	Mixed granules	5.6-7.4	Gram-negative bacteria
Bactericidial/permeability-increasing protein (Mr=60,000)	Azurophilic granule	7.0	Gram-negative bacteria
Defensins (Mr<3500)	Azurophilic granule	7.0-8.0	Gram-positive and gram-negative bacteria, *C. neoformans,* herpes simplex virus, type 1
Azurophil-derived bactericidal factor (Mr=29,000)	Azurophilic granule	5.5	Gram-positive and gram-negative bacteria

From: Forehand,J.R., Nauseef, W.M., Johnston, R.B.: "Inherited disorders of phagocyte killing " in "The metabolic basis of inherited disease", Scriver, C.R., Beaudet, A. L., Sly, W.S., Valle, D., (Editors), sixth edition, vol II, pag. 2779. 1989

2.2 *Oxygen-dependent mechanisms*

Among this group, two different mechanisms have to be mentioned: the so called 'respiratory burst' and the myeloperoxidase system. The respiratory burst is the enzymatic pathway impaired or completely abolished in CGD patients.

Respiratory Burst.

During phagocytosis, neutrophils undergo a burst of oxidative metabolism (18), (130), (16), (137), (13), (130). This event begins with a great increase in oxygen consumption as well as in the utilization of glucose via the hexose monophosphate shunt. The process ends with the production of the bactericidal oxygen metabolites superoxide anion (O_2^-) (15), (126), hydrogen peroxide (H_2O_2) (136), hydroxyl radical (·OH) (126), (136), (277), (308), and, perhaps, singlet oxygen. (6), (150). This cyanide-insensitive (250) increase in oxidative metabolic activity is commonly named the "respiratory burst".

The enzymatic complex responsible for the respiratory burst is called NADPH oxidase. The oxidase system is composed of a cellular membrane-associated complex (cytochrome b_{558}) and at least two cytosolic components. It is present only in phagocytic cells. The complex reduces oxygen univalently using NADPH as electron donor (13) (figure 1):

$$2O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} 2O_2^- + NADP^+ + H^+$$

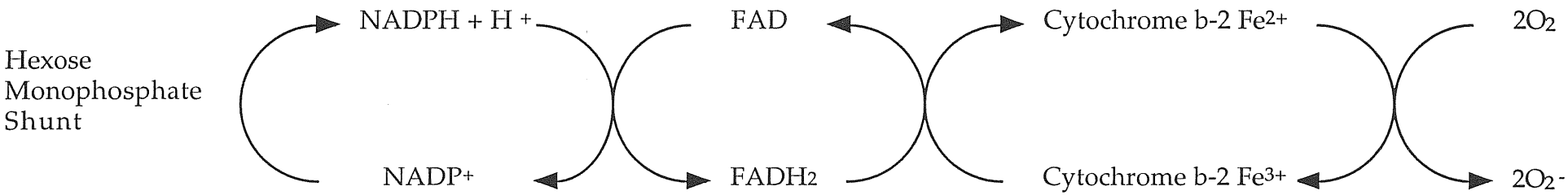


Figure 1. Proposed reactions of the superoxide generating oxidase of phagocytes.

Most of this O_2^- is thought to react with itself in a dismutation reaction (either spontaneously or more rapidly in the presence of superoxide dismutase) to form the second product of the respiratory burst, H_2O_2:

$$2O_2^- + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

The list of oxygen metabolites generated in the phagocytosis-dependent respiratory burst includes OCl^- ((139) and hydroxyl radical (·OH) (126), (277), (307), (308).·OH is a highly potent oxidant formed by the interaction between O_2^- and H_2O_2 in the presence of iron or other metals (Haber-Weiss reactions); this reactions is summarized as follows:

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + \cdot OH$$

Another possibility for ·OH production is the interaction between H_2O_2 and iron (Fenton reaction) (13).

Other oxidants have also been described. They are formed by the reactions of hypochlorite with ammonia or amines and hence named chloramines (280).

The formation of all these bactericidal agents, depends on the production of O_2-. In other words, the formation of O_2^- is the key event in the respiratory burst. The impairment of the production of this metabolite is responsible for the inability of phagocytic cells to produce toxic agents for the destruction of ingested bacteria.

Myeloperoxidase-catalyzed system.

Myeloperoxidase (MPO) is located in the azurophilic granules of neutrophils and in the primary lysosomes of monocytes. It is biochemically and immunologically distinct from eosinophil peroxidase (248). Stimulated neutrophils release the products of the respiratory burst and the contents of the granules, including MPO, into the phagolysosome or in the extracellular space. The combination of MPO, H_2O_2, and halide ions (the MPO-H_2O_2-halide system) results in the production of hipohalous acid and other intermediates that produce cytotoxicity. Hypochlorous acid and the monochloramines, the long-lived oxidants derived from HOCl, have been best studied in this regard (98), (106). The actual mechanisms for the cytocidal activity have not been established, but some possibilities include destruction of bacterial electron transport, (5) ablation of the bacterial adenine nucleotide pool, or oxidation of iron and sulfur centers critical for bacterial viability (242), (241).

Due to the fact that all the oxygen metabolites produced by the respiratory burst are potentially very dangerous for the cell, a number of protective mechanisms have been developed. Products of the respiratory burst are released into the phagolysosome where they participate in the destruction of the ingested particle. As toxic oxygen metabolites can damage other circulating cells and adjacent tissues as well as the stimulated phagocyte, it is important to concentrate the site of action in the phagolysosome. For these reasons cells have developed different antioxidant mechanisms that are mainly represented by superoxide dismutase, glutathione, catalase, vitamin E and vitamin C.

Superoxide dismutase, which is found in the cytoplasm as a copper-zinc-containing enzyme and in the nucleus as a manganoprotein, is the

principal scavenger of O_2^- (175), (67), (179). This enzyme catalyzes the conversion (dismutation) of two O_2^- molecules to H_2O_2 and oxygen.

Glutathione is a tripeptide found in all tissues and serves as a substrate for H_2O_2 in a reaction catalyzed by glutathione peroxidase (239).

Catalase enzymatically converts H_2O_2 in H_2O and oxygen (40), (240).

Vitamin E (α-tocopherol) reacts with toxic oxygen radicals and preserves cell membranes from oxidative damage (163), (14).

Vitamin C (ascorbic acid) combines with oxygen free radicals to form harmless by-products (14), (26) and can react with vitamin E radicals to regenerate vitamin E (205).

3. Components of the superoxide-generating oxidase of phagocytic cells

NADPH oxidase is a multiple-component enzyme, consisting of at least five subunits. Two of these subunits are integral membrane proteins that constitute the flavo-heme protein cytochrome b_{558}: they are called gp91-*phox* (p from protein and *phox* from *ph*agocyte *ox*idase) and p22-*phox*, due to their molecular mass. The cytochrome b is called 558 since it displays an absorption band at 558 nm; it is also called -245 because of its midpotential, i.e. the point at which it is balanced between oxidation and reduction. The other three subunits are localized in the cytosol of resting phagocytes and are called p47-*phox*, p67-*phox* and Rac-1 (GTP-binding protein). In activated phagocytes, the cytosolic components translocate to the cellular membrane conferring enzymatic activity to cytochrome b_{558} . The five proteins mentioned before are sufficient to produce superoxide in a cell-free system containing oxygen, GTP, and SDS or arachidonic acid as activator of the oxidase (245). In intact cells, additional proteins are probably required for activation and deactivation of NADPH-oxidase (1), (185), (152). In particular, a 40 kDa protein as well as another GTP-binding protein termed Rap 1A may take part to the complex regulation system that control NADPH oxidase activity.

Cytochrome b_{558} components, p47-*phox*, p67-*phox* and p40-*phox* will be described in the following paragraph while Rac-1 and Rap1A proteins will be described in the paragraph on the "Physiological aspects of superoxide-generating oxidase".

3.1 Membrane components: gp91-phox and p22-phox

gp91-*phox* core protein

The core protein of the gp91-*phox* protein is composed by 571 amino acids as it can be deduced by the cDNA sequence (246). Using anti-peptide antibodies it has been possible to map the protein fragment exposed on the cell surface (121). One region was found between residues 150 and 172, close to two possible glycosylation sites (Asn 132 and 148). Another region was localized between residues 369 and 398. Both of these regions exhibit high hydrophilicity in accordance to their surface exposed position (figure 2).

The region 399-421, immediately after the second hydrophilic region, is hydrophobic and considered to be a transmembrane segment. The carboxyl-terminal stretch is mostly hydrophilic and it is likely to be exposed in the cytoplasm, since it is sensitive to papain when a membrane preparation is subjected to digestion. It probably interacts with cytosolic proteins necessary for the O_2^- generating activity. Another possible membrane-spanning region is localized between residues 168-190 close to the cell surface exposed region 150-172. There are other residues (6-25, 46-76 and 210-225) that are also hydrophobic. These regions may be transmembrane as well, or may be sites for specific interaction with the small subunit (p22-*phox*) of the cytochrome. Incidentally, His-100, -110, -117, -207, and 220 are in the hydrophobic regions and are spaced similarly to the heme-coordinating histidines in other cytochromes (246). The above reported features are schematically summarized in the model of figure 2.

Further analysis of the COOH-terminal segment of the protein were conducted with synthetic peptides. Rotrosen (244) demonstrated that the

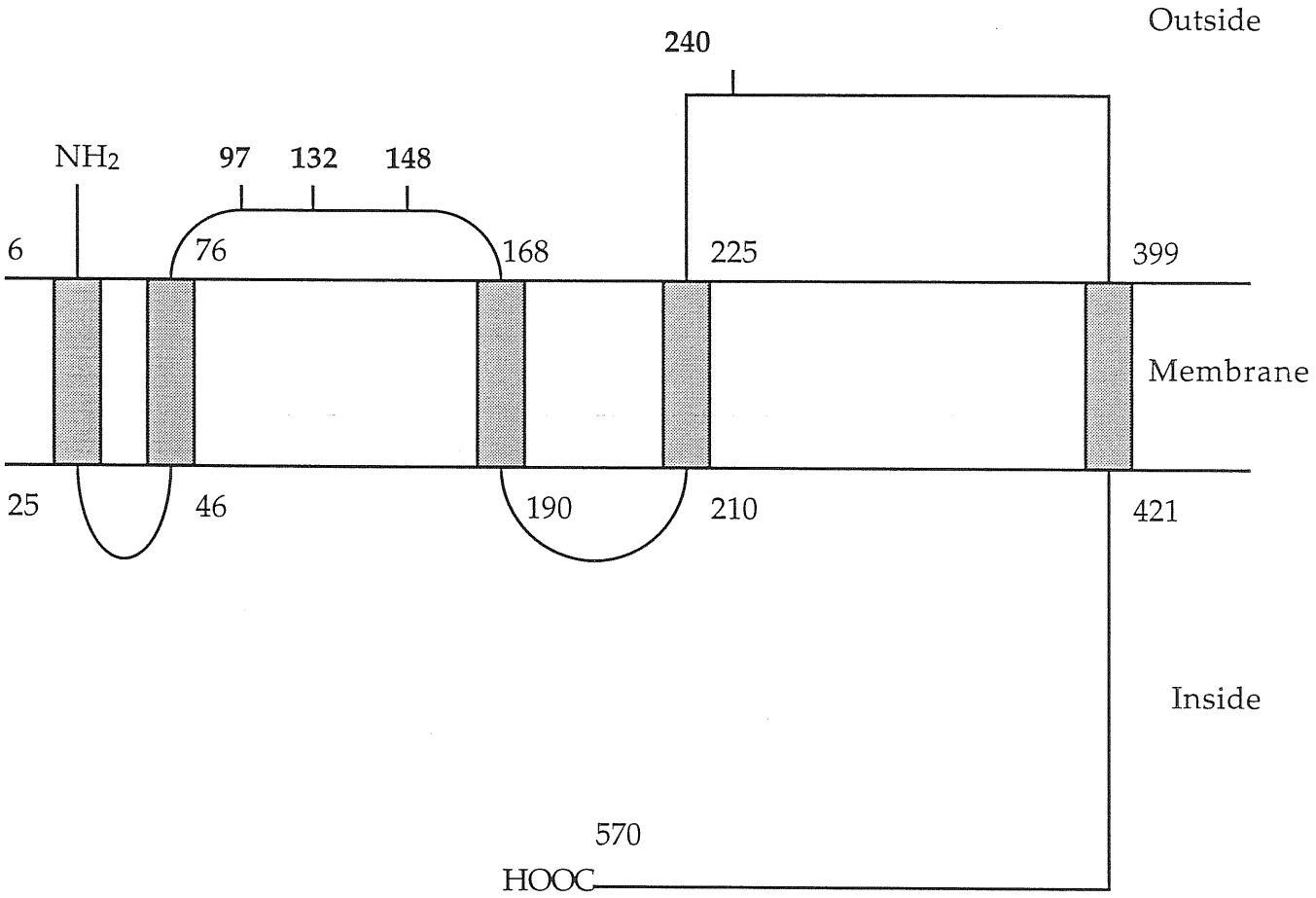


Figure 2. Schematic representation of gp91-*phox*. Regions 6-25, 46-76, 168-190, 210-225 and 399-421 are hydrophobic and are considered to be transmembrane segments. Asn 97, 132, 148, 240 represent possible glycosylation sites.

region 559-565 can be a functional domain of gp91-*phox*. Synthetic peptides encompassing a 7-amino acid sequence (559-RGVHFIF-565) of the carboxyl-terminal region of the gp91-*phox* were used in a cell-free O_2^- generation system. The addition of the peptide in the system was able to block superoxide anion production. Inhibition was concentration-dependent and occurred only when the peptide was added before activation by arachidonate. These results suggest that the peptide specifically inhibits processes critical to oxidase activation. It was also seen that the synthetic peptide was able to inhibit p47-*phox* phosphorylation. The simplest model that can account for both inhibition of oxidase activity and the inhibition of p47-*phox* phosphorylation is the one which assumes that, in binding to p47-*phox*, the RGVHFIF-containing peptides prevent p47-*phox* phosphorylation by blocking access to an arachidonate-activated kinase. This means that the 559-565 COOH-terminal segment of the large subunit mediates assembly of the oxidase components.

Further analysis of the peptide (141) demonstrated that the inhibitory effect of the 559-565 region is mainly due to Arg-559, Val-561, Ile-564 and both Phe-563 and 565. On the contrary, Gly-560 and His-562 can be substituted with Ala with little loss of inhibitory activity. The requirement of Val, Ile and both Phe for inhibitory activity in the cell-free assay suggests that binding of native gp91-*phox* carboxyl terminus to another oxidase component is mediated in part by hydrophobic interactions within the binding site. The importance of Arg in inhibitory activity suggests that there is a critical ionic interaction between the native gp91-*phox* carboxyl-terminus Arg and a polar amino acid within the binding pocket of the oxidase component that binds to gp91-*phox*.

Other information about gp91-*phox* can be deduced from alignment analysis of the amino acid sequence. Alignment with several flavoproteins reveals significant similarities. In particular, two gp91-*phox* regions similar to the FAD binding site of

human glutathione reductase and related enzymes and one similar to ferredoxin and related enzymes have been found (275) as detailed as follows.

1) There is similarity in the sequence of residues 218-223 (Gly-X-Ala-X-X-Gly) of gp91-*phox* with that of the canonical ADP binding fingerprint (Gly-X-Gly-X-X-Gly) found in glutathione reductase and related enzymes (311), (83). In particular, the putative ADP binding fingerprint in gp91-*phox* is identical to that of ATCC 9790 NADH peroxidase (182). It is also possible to notice that the sequence around the fingerprint conforms reasonably well to those for the ADP-binding βαβ fold of several flavoproteins (83), and that the Chou-Fasman algorithm (47) predicts that this region of gp91-*phox* has high probability of forming a βαβ structure. From these data it can be concluded that the portion of residues 214-246 forms a FAD binding βαβ-fold.
2) The second region of similarity can be found in segment 350-360. Similar regions in FAD-binding enzymes form a β-sheet structure that turns the aspartate: this acidic amino acid is absolutely conserved and forms a hydrogen bond with the O3'-hydroxyl group of the ribityl moiety of FAD (83). In the gp91-*phox* protein, besides the conservation of aspartate, the Chou-Fasman algorithm predicts that this region has a high probability of forming a β-sheet.
3) The region 335-345, adjacent to the β-sheet binding ribityl chain of FAD, exhibits some similarity to the portion binding the isoalloxazine ring of the flavin moiety of FAD found in ferredoxin reductase (131) and related enzymes (35). This data also support the conclusion that this region is involved in FAD-binding.

   Taken together, these observations indicate that the middle portion of gp91-*phox* is likely to form a FAD-binding domain.

Other interesting similarities can be found between gp91-*phox* and some NADP(H)-dependent flavoenzymes (275). In particular, there are two regions in the COOH-terminal portion of gp91-*phox* homologous to the NADPH-binding domains of the FNR family and one homologous to enzymes closely related to cytochrome P-450 reductase.

1) The sequence 405-423 of gp91-*phox* shows a strong homology with the so called glycine-rich region of the FNR family and contains the conserved motif Gly-X-Gly-X-X-Pro. This region is involved in binding to the pyrophosphate moiety of NADPH (131).
2) The second homologous region is the one between 531-546, showing the conserved Cys-Gly dipeptide: this fragment is proposed to be the binding site for the nicotinamide mononucleotide moiety of NADP(H) (131), (35), (119), (222).
3) The region between residues 441-450 of gp91-*phox* shows homology with enzymes closely related to cytochrome P-450 reductase, especially to cytochrome P-450$_{BM-3}$. In the homologous segment, Cys 566 is included in the NADPH-binding site (292), (104); the corresponding amino acid of gp91-*phox* (Cys 445) is conserved.

In conclusion, these data altogether indicate that the NH_2-terminal portion of gp91-*phox* is likely to be important for binding to the heme and for the formation of a stable complex with p22-*phox* while the middle and the COOH-terminal portions are involved in FAD binding and NADPH-binding respectively (figure 3). The COOH-terminal segment seems also to play a critical role in the interaction with the cytosolic components of the oxidase complex. The FAD-binding site homology with glutathione reductase and its related enzymes, and the NADPH-binding site homology with the FNR family, suggest that the gp91-*phox* gene probably has arisen through a fusion of the ancestral genes for these two distinct types of flavoproteins (131), (38).

gp91-*phox* glycosylation pattern

The large subunit of cytochrome b_{558} is a heavily glycosylated protein with a relative molecular mass of about 91kDa. In neutrophils four possible glycosylation sites have been described: these are represented

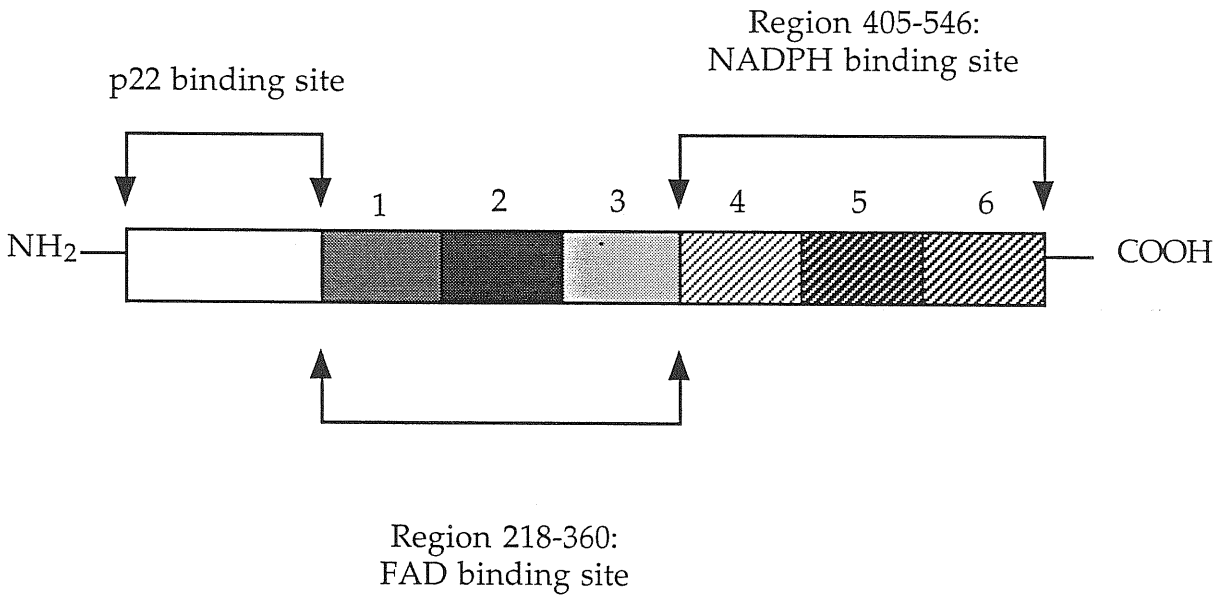


Figure 3. gp91 *phox* functional domains. Regions 1 and 3 of gp91-*phox* are similar to the FAD binding site of human glutathione reductase; region 2 is similar to the FAD binding site of ferrodoxin reductase. Regions 4 and 6 show similarities with the NADPH-binding domains of the FNR family; region 5 has homology with the NADPH-binding site of P-450 reductase.

by Asn 97, 132, 148 and 240. Only N-linked and no O-linked oligosaccharides are present on the core protein purified from neutrophils (212), (105). The most abundant monosaccharides are represented by N-acetylglucosamine and galactose (105).

Various glycosylation patterns can be found in different phagocytic cells type (142): the bands that can be resolved on a SDS-PAGE immunoblot analysis span from 78 to 93 kDa in neutrophils, from 74 to 115 kDa in eosinophils, from 82 to 99 kDa in monocytes, from 77 to 110 kDa in DMSO-induced HL-60 cells. However, after complete digestion with endoglycosidase F, the core peptide has the aspected molecular weight of 55 kDa in all phagocytic cell types. This means that different enzymatic pathways, responsible for the post-translational glycosylation, act in different phagocytic cells. This is common to many other proteins with widespread tissue distribution (108).

The N-linked oligosaccharides contribution to superoxide anion generation is not well understood. However, it is unlikely to play a critical role (142) since a cytochrome b_{558} complex deficient in N-linked oligosaccharides may be functionally competent to mediate the respiratory burst, although at a lower level.

p22-*phox*

This protein is composed of 195 amino acids and is characterized by a proline-rich COOH-terminus (27% of the 63 COOH-terminal residues). The primary structure, as it can be deduced from its cDNA sequence (212), suggests that it is a membrane protein. It is possible to obtain some information about its structure and its position on the cellular membrane using antibodies. Imajoh et al. (121) showed that the NH_2-terminal and the COOH-terminal portions of the protein face to the cytoplasmic side of

granulocyte membranes. Antibodies raised against the two regions can recognize the specific epitopes only after neutrophil permeabilization. The COOH-terminal sequence 'PPSNPPPRPP' (aa. 150-159) has been proposed to be a possible binding site for the SH3 domain of one of the cytosolic proteins (310). On the contrary, region 49-62 is probably extra cellular (191), since monoclonal antibodies against this domain react on the outer surface of phagocytes.

Interesting data can be obtained by alignment analysis. Structural motifs in common with other heme-containing proteins have been described (212). In particular, a 31-residue region containing His-94 is 39% identical to a corresponding histidine-bearing region of polypeptide I of mitochondrial cytochrome c oxidase. Another interesting feature of p22-*phox* is the overall hydropathy plot resembling that of myoglobin, with a greatly increased hydrophobic environment for one of the potential Fe coordinating sites of heme at His-94. This histidine residue aligns exactly with an iron-coordinating histidine of myglobin. For this reason it can be concluded that His-94 is involved in heme group binding.

Secondary structure predictions by the method of Chou and Fasman (47) suggest that the polypeptide is highly flexible, particularly near its COOH-terminus and that it contains at least three α-helical regions. A schematic representation of the protein model is given in figure 4.

Cytochrome b_{558} heme content

The theoretical heme content for a cytochrome b heterodimer of aggregate molecular mass of 113 kDa (91+22kDa) containing only one heme is of 8.9 nmol/mg of protein (224). However, the measured heme

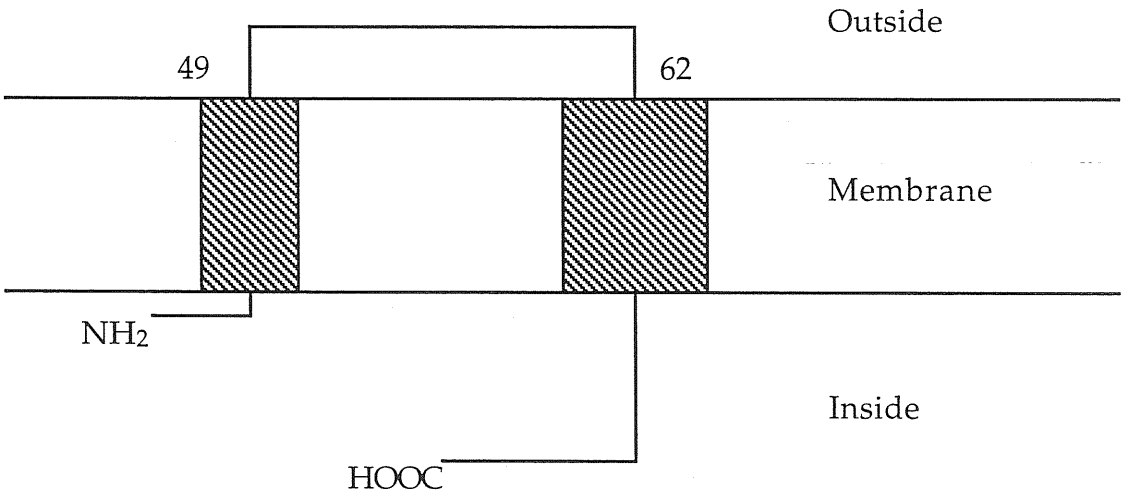


Figure 4. Schematic representation of p22-*phox*. Region 49-62 is considered to be an extracellular segment.

content of the purified cytochrome b is 20-30 nmol/mg of protein depending on the value used for the extinction coefficient (210), (216). Therefore, it is possible to conclude that cytochrome b is a multi-heme protein. Evidences supporting this speculation come from the spectral studies conducted by several authors (120), (118), (285), (224). These authors demonstrated that human neutrophil cytochrome b is a bi-heme or possibly a tri-heme molecule with at least one heme residing in gp91-*phox* protein, and one shared between both subunits and that the heme-containing regions of the cytochrome probably lie within the membrane lipid bilayer. This structure is common to other b-type cytochromes from different systems: in chloroplast and in bacteria the hemes are present in the membrane bilayer and shared between two different polypeptide chains of the cytochrome molecule (99), (111), (12), (309), (276), (69). Such a multi-heme structure would be consistent with an electron transfer function for the cytochrome-b by providing an efficient mechanism for transferring electrons across the plasma membrane to the extracellular surface where oxygen could be reduced to create superoxide.

One peculiarity of cytochrome-b lies in its six coordination sites. All known heme-containing enzymes involved in reduction or activation of oxygen have a heme in which the five coordination site are occupied by intrinsic ligands, and the sixth coordination sites is opened for binding to oxygen or other extrinsic ligands. On the contrary, it seems that the heme of cytochrome b_{558} is a low spin six-coordinate structure according to optical absorption (120), (122), resonance Raman (118) and EPR (256), (180) spectroscopy. The strong binding of all the axial ligands to the heme iron is further confirmed by no effects of respiratory inhibitors such as CO. All these results strongly suggest that an electron is directly transferred from the heme of cytochrome to O_2 without ligation of O_2 to the heme iron during the catalytic cycle.

3.2 *Cytosolic components*

p47-*phox*

From the p47-*phox* cDNA sequence (296) it is possible to deduce a protein of 373 amino acid with a molecular mass of about 42 kDa (figure 5). Analysis of the derived amino acid sequence demonstrates a protein having four major structural features of potential interest.

1) The protein has an NH_2-terminal glycine residue (Gly-1) that could potentially serve as a site for myristolylation (296). The NH_2-myristoylation is an early event in acylprotein biosynthesis. A variety of observations (283) suggest that this process may participate in the juxtapositioning of the acylprotein and other components of cellular regulatory circuits. This might occur either by directing the acylprotein to particular cellular membranes, or by permitting it to interact with polypeptides that reside either in the cytoplasm or membranes. In the case of p47-*phox*, the myristoylation could allow protein translation to the membrane during phagocyte activation. However, it has been noticed (283) that Asp-3, Ile-6, Arg-7 and Ile-9 may inhibit the attachment of myristic acid to the N-terminal glycine. Therefore, it remains debatable whether Gly-1 is a real myristoylation site.

2) The COOH-terminal third of the protein is a serine-rich region (13%) containing four segments with six serine residues in configurations that are favourable to be potential sites for phosphorylation by serine and threonine protein kinase (82). Another potential site is represented by Tyr-97. These sites are in accordance with previous reports of a 47 kDa component of the

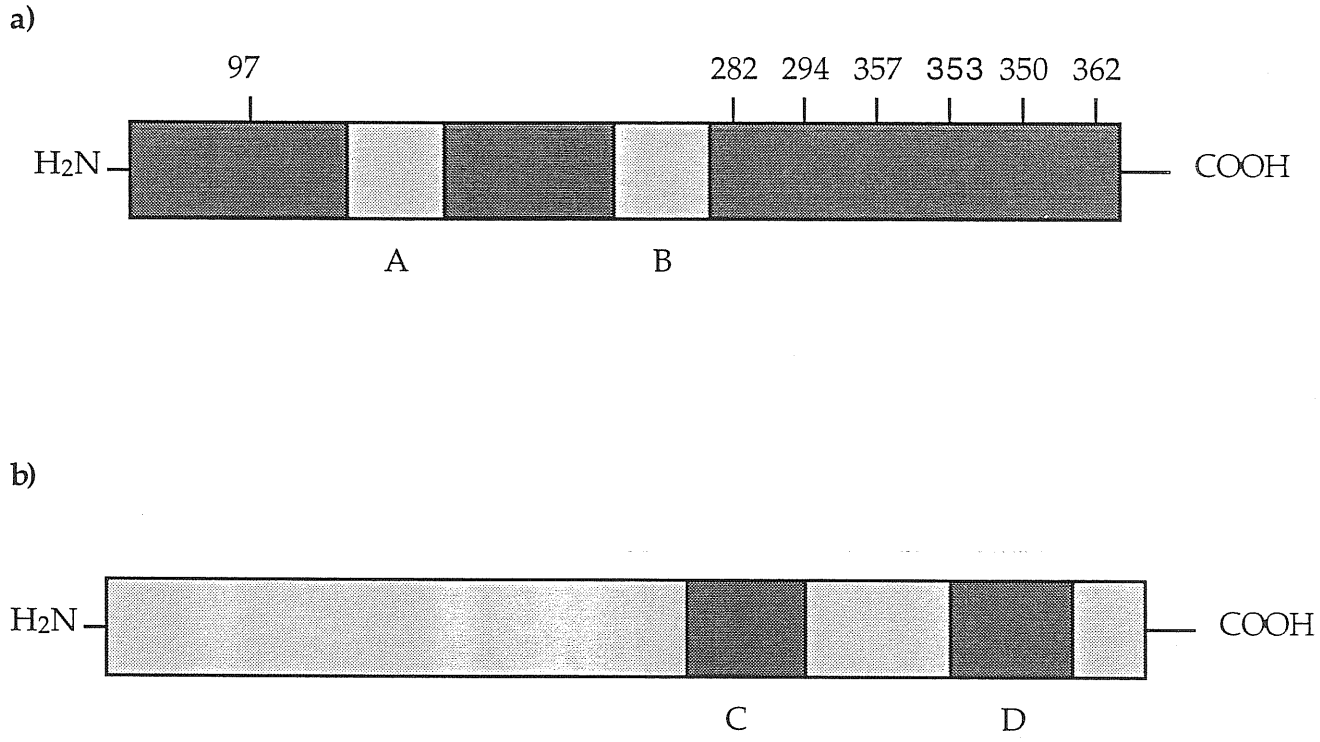


Figure 5. Schematic representation of p47-*phox* **(a)** and p67-*phox* **(b)**. The numbers indicate the possible phosphorylation sites (Ser 282, 294, 350, 353, 357, 362 and Tyr 94). The segment A (161-211) and B (231-281) in panel **(a)**, C (245-295) and D (462-482) in panel **(b)** represent the portions of the two protein homologous to the putative regulatory regions of the src superfamily (SH3).

NADPH oxidase that appears to be a substrate for phosphorylation (255), (37), (33), (200), (199).

3) Two of the three GTP-binding domains already described (71) are present in the p47-*phox* protein. The p47-*phox* sequence AGGSSGK (residues 262-268) matches the described sequence GXXXXGK, except that it contains the less common A in the first position. It represents the first element that might be involved in the interaction with the phosphate portion of the GTP molecule. The second GTP binding domain present in p47-*phox* is DITG (residues 151-154) which matches the described sequence DXXG. Also this fragment is involved in binding the phosphate residues of the GTP molecule. It is important to point out the fact that the third region required for the GTP-binding domain (NKXD) is not found in p47-*phox*.

4) By comparing the p47-*phox* sequence with all the known sequences in data bases, it is possible to notice that the middle portion of p47-*phox* contains two regions (161-211 and 231-281) of significant homology to one of the putative regulatory regions of proteins belonging to the src superfamily, named SH3 (214). The first regions has 29% homology with the SH3 domain, while the second 24%. Besides the non receptor tyrosine kinases, these domains are found in a number of other cytoplasmic proteins (174), (231), (272), including the 67-kDa cytosolic NADPH oxidase factor. It has been proposed that the SH3 domain of p47-*phox* is involved in protein association with the inner part of the plasma membrane (232).

Recently (156), a direct binding between p47-*phox* and p22-*phox* has been demonstrated.

p67-*phox*

The nucleotide sequence of the p67-*phox* cDNA (158) predicts a 526-amino acid protein with a molecular mass of 61 kDa. By comparing the deduced amino acid sequence of p67-*phox* with the sequences contained in the NBRF database, it is possible to notice significant structural similarities between the p67-*phox* COOH-terminal domain (462-482) and a limited portion of the non catalytic SH3 region of src superfamily. A second repeat of the SH3 domain (29% similarity) is present within the mid segment of p67-*phox* (residues 245-295) (figure 5). Although the SH3 domain has been detected in many proteins together with additional non catalytic segments of src (regions SH2 and B) (174), (302), (274), (293), (231), only the SH3 region has been found in p67-*phox*, p47-*phox*, myosin I and α-spectrin, suggesting that this region may function independently of the other two src-like regions B and SH2. One possible explanation for the action of the SH3 domain in p47-*phox* and p67-*phox* comes from the study of the myosin I protein. In this case, the SH3 domain is located close to a proline rich region in the COOH-terminal domain: a similar proline-rich sequence together with SH3 motifs is present in both p47-*phox* and p67-*phox*. This observation suggests that p47-*phox* and p67-*phox* could bind directly to actin (158).

p40-*phox*

Recently (310), a new cytosolic component of the NADPH oxidase activation complex has been identified. The amino acids sequence obtained from its cDNA reveals a protein of 339 amino acids. The

similarity between p40-*phox* and the other two cytosolic components is very interesting. In particular, the similarity with p47-*phox* is intriguing : the two proteins have 22% identity over 245 amino acids and if we take into account conservative substitutions the similarity is 67% over the NH_2-terminal 70% of their sequence. p40-*phox* also contains a region (175-225 residues) having similarity with SH3 domains of a number of proteins among which the COOH-terminal SH3 domain of p67-*phox*. Due to the fact that the SH3 domain seems to bind to proline-rich sequences (229), it was argued that p40-*phox* may bind to both p47-*phox* and p67-*phox*. This hypothesis is supported by the observation (310) that all three proteins translate to the plasma membrane upon activation of the oxidase. If we consider the fact that the COOH-terminal portion of p22-*phox* is very proline-rich, we can imagine a model where the cytosolic complex (p40-p47-p67) migrate towards the membrane where it may bind to the membrane component of the NADPH oxidase. However, it must be mentioned the fact that in an artificial cell-free assay the presence of p40-*phox* is not necessary for activation of the oxidase: its action may be replaced or circumvented by SDS or arachidonic acid as activator.

4. Physiological aspects of superoxide-generating oxidase

Even if all the steps involved in superoxide generation in intact phagocytes are not known, it is possible to describe the general events required in this complex enzymatic reaction (figure 6).

The respiratory burst in phagocytes initiates after (1) ligand binding to surface receptors (see below). The consequence of this binding is the induction of conformational changes in the receptors and subsequent coupling of these receptors to membrane-bound trimeric GTP-binding proteins. This leads to the formation of a (2) second messenger (inositol phosphates and diacylglycerides) which is able to activate (3) cellular kinases which in turn (4) are able to phosphorylate their substrates (among which p47-*phox*). (5) The Rac and Rap 1A activation is another event required for triggering NADPH-oxidase. (6) The migration of the p47-p67-p40 protein complex towards the membrane components and the consequent activation of the oxidase with the production of superoxide, ends the process.

These events are detailed in the following paragraphs.

4.1 Early steps in superoxide production

Unstimulated neutrophils consume relatively little O_2 (less than 1 nmol $O_2/10^7$ cells at 37 °C). Within few seconds after contact with specific stimuli, the rate of O_2 consumption abruptly increases by a factor of 50-100 (186). Stimuli that are present at the inflammation sites are represented mostly by opsonized microorganisms, complement fragment C5a (which is formed upon complement activation after interaction of microorganisms with antibodies), N-formylated methionylpeptides (that

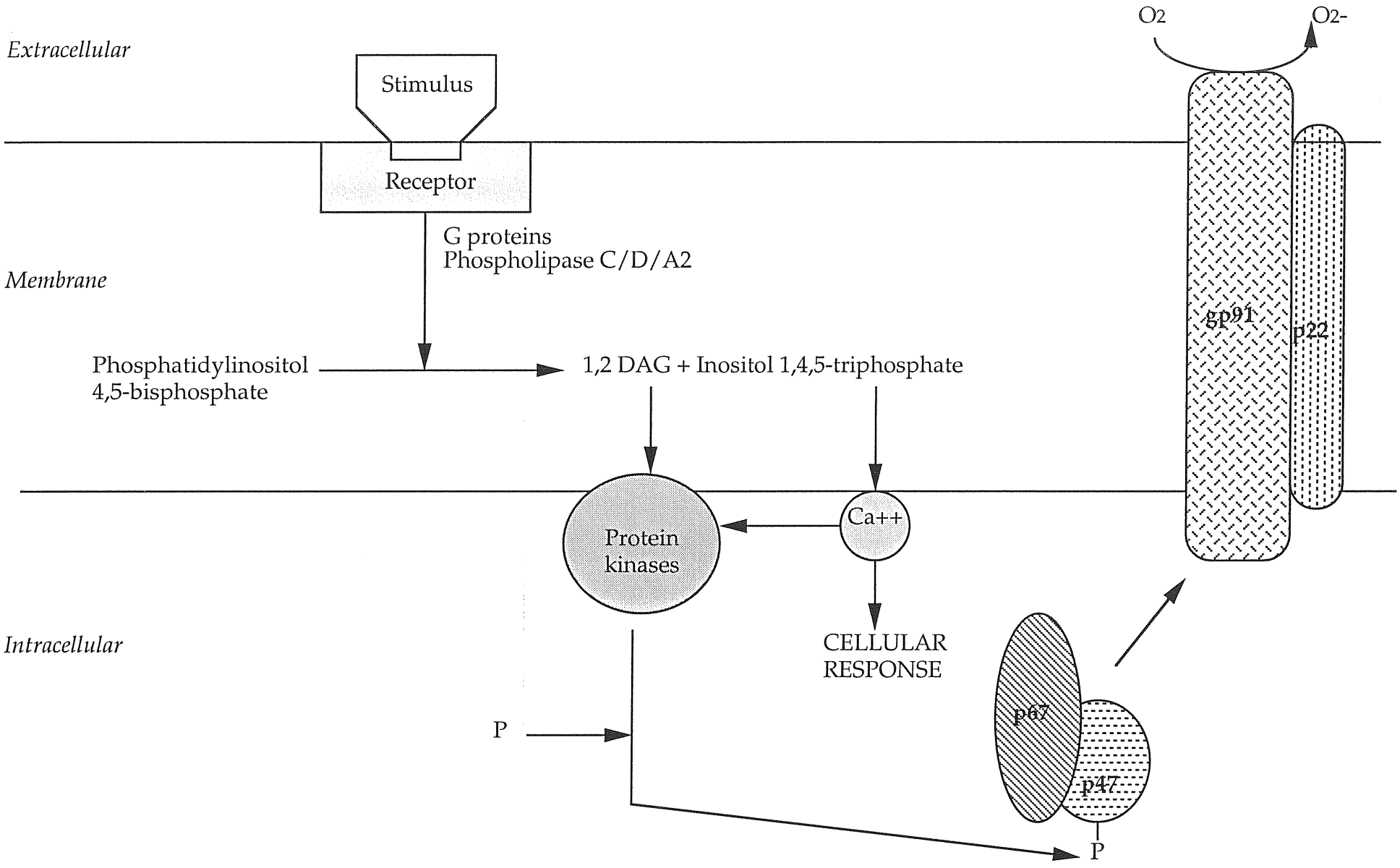


Figure 6. Schematic representation of NADPH-oxidase activation.

can be released by lysis of dead microorganisms or secreted by bacteria), and two bioactive lipids produced by activated cells, namely platelet activating factor (PAF) and leukotriene B4 (268). Other more recently discovered specific stimuli are represented by neutrophil activating protein 1 (NAP 1) (17), melanoma growth-stimulatory activator (188), and neutrophil activating protein 2 (NAP 2) (299).

All these ligands act via distinct surface receptors. They are able to trigger respiratory burst as well as chemotaxis. One relevant difference between the two actions lies in the fact that lower concentrations of stimuli are required for triggering chemotaxis as compared to the concentration for production of O_2^- (186). For example, the concentration of fMet-Leu-Phe necessary to activate chemotaxis is around 10 nM, while that required for stimulation of the respiratory burst is higher than 100 nM.

Other not physiological stimuli can induce the respiratory burst. Among these, fluoride (61), 4β-phorbol 12-myristate 13-acetate and some other phorbol diesters (230), A23187 (23) and opsonized zymosan (36). The time required between the interaction of the stimuli with the specific receptor and the superoxide production is less than 5 sec. (317). The magnitude and the duration of the respiratory burst depend on the nature and the amounts of the agonists used and also on the state of neutrophils prior to stimulation. Neutrophils can exist in a resting, activate and primed state. Priming has been individualized as a step distinct from activation since it does not induce superoxide generation, but reduces the lag before the onset of O_2^- production and amplifies O_2^- generation in response to agonists (127), (107), (298).

Despite the number of different agonists able to trigger the respiratory burst, there seems to be a common mechanism for neutrophil activation upon ligand binding. This mechanism involves a G protein,

sensitive to *Bordetella pertussis* toxin, termed G_N (267) and results in the increase in the internal concentrations of inositol triphosphate and diacylglycerol, in the release of Ca^{2+} from intracellular stores, in the stimulation of respiration and in the secretion of granular enzymes (290), (198), (34). The G_N protein is distinct, both biochemically and immunologically, from other G proteins sensitive to the pertussis toxin (among which Gi, the guanosine-nucleotide-binding inhibitory subunit coupled to adenylate cyclase, transducin and Go, the guanosine-nucleotide-binding "o"subunit found in brain (94), (201), (97).

4.2 Production of a second messenger able to activate protein kinase C

The agonists of the respiratory burst are able to induce the activation of a G_N protein which in turn is able to activate three different phospholipases: phospholipase C, phospholipase D and phospholipase A_2. They all contribute to the synthesis of a second messenger by an intricate set of reactions ending up with the activation of protein kinase C (PKC) and possibly other not yet well characterized kinases. The signals that determine the contribution of each of the phospholipases remain to be determined.

Phospholipase C

Many authors (51), (198), (265), (264), (53), (273) have demonstrated that agonists are able to activate a phosphatidylinositol-4,5-bis phosphate ($PtdInsP_2$) specific phospholipase C through a G_N protein. The consequence of the phospholipase C action is the cleavage of $PtdInsP_2$ with the production of 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($InsP_3$) (284). DAG and $InsP_3$ are two activators of protein kinase C. DAG

acts directly while $InsP_3$ indirectly through a rapid release of Ca^{2+} from non-mitochondrial stores, probably through a specific receptor (269). The rise in the concentration of free cytosolic Ca^{2+} seems to play a critical role in superoxide generation, as it can be deduced by the fact that neutrophils depleted in Ca^{2+} fail to produce O_2^- in response to fMet-Leu-Phe (160). Furthermore, it has been demonstrated that the rise in the free cytosolic Ca^{2+} always precedes the onset of the respiratory burst (297).

Phospholipase D

It is known (279), (9) that diacylglycerol can be formed not only by the phospholipase C pathway, but also by an indirect pathway involving phospholipase D. Phosphatidylcholine is hydrolysed by phospholipase D to phosphatidic acid which in turn is degraded by a phosphatidic acid hydrolase to diacylglycerol that is a well known activator of PKC.

There are many experimental evidences supporting the hypothesis of a real contribution of phospholipase D in activating the respiratory burst. First of all it has been demonstrated, using a radio-labeled lipid precursor of phosphatidylcoline, that diacylglycerol is generated directly by phospholipase D in activated HL60 granulocytes (4), (27), (206); further studies confirmed this result in human neutrophils (27), (93), (189), (190). Another line of evidence is represented by the good correlation between the levels of phosphatidic acid in activated neutrophils and the release of O_2^- (146).

More recently, it has been proposed (183) that phosphatidic acid (PA) is able to directly activate NADPH oxidase independently of PKC activation since the use of PKC inhibitors and of PA phosphohydrolase does not inhibit O_2^- production. According to these results, PA seems to act at a site downstream of PKC.

Phospholipase A_2

The activity of phospholipase A_2 is upregulated during phagocyte oxidase activation (172), (21), (153), (128), (52) as it can be estimate by the production of fatty acids and, in particular, of arachidonic acid. Furthermore, it has been demonstrated that a variety of phospholipase A_2 inhibitors are able to turn off the O_2^- generation system (110). However, it must be mentioned the fact that of the two forms in which phospholipase A_2 exists, only the one bound to the plasma membrane increases its activity after neutrophil stimulation (19).

The regulation of this phospholipase gives us an idea of the complex relations in this network of biochemical reactions. In stimulated neutrophils, activation of the phospholipase A_2 appears to be under the control of Ca^{2+} concentration and of a G-protein (54), (52). The rise in cytosolic Ca^{2+} may be subsequent to phospholipase C activation, via the effect of $InsP_3$ on Ca^{2+} stores.

4.3 Protein kinases involved in respiratory burst activation

PKC

PKC catalyses the phosphorylation of serine and threonine residues in specific proteins. PKC exists in multiple isoenzymes but only the β and α isoforms are present in human neutrophils: the α isoform is largely predominant (219), (164).

The concept of PKC-dependent pathway for NADPH oxidase activation is mainly based on two sets of experimental evidences. The first concerns the study of the effect of formylpeptides and phorbol esters on

superoxide production and on the incorporation of (^{32}P)Pi: a correlation between the level of oxidase activity and the degree of phosphorylation of p47-*phox* (252) was found. It was also demonstrated that p47-*phox* is a substrate of PKC (238), (192), (148). The second set of experiments is based on the use of an agonist or an antagonist of PKC activity in relation with the respiratory burst (57), (312). These data are in favour of a relevant role for PKC in the activation of respiratory burst.

Although these data seem to prove a central role for PKC in superoxide generation, this is probably not the only pathway involved.

Other Kinases

Recently, it has been proposed (78) that several uncharacterized protein kinases are also able to phosphorylate p47-*phox*. In particular, it has been demonstrated that, upon stimulation of human neutrophils with PMA, the activity of two kinases of 96 and 105 kDa is greatly increased. It was proposed that these kinases undergo covalent modification (phosphorylation) that increase their catalytic activity (84). The molecular mass of the two kinases is absolutely different from that of PKC (84 kDa) and PKA (38 kDa catalytic subunit) (84). According to these data, it is possible to conclude that the 96 and 105 kDa kinases may represent new enzymes that can use p47-*phox* as a substrate. These data are in favour of the idea that extremely intricate networks of reactions are involved in the control of p47-*phox* phosphorylation and, as a consequence, of superoxide generation.

4.4 p47-phox, p67-phox and p40-phox translocation

As discussed above, in PMA stimulated neutrophils the phosphorylation of p47-*phox* seems to play a relevant role in activation of respiratory burst (192). It has been suggested that phosphorylation occurs in two subsequent steps (203), (114), (244). The first phosphorylation takes place in the cytosol; then the phosphorylated protein translocates to the membrane and binds, through its tyrosine-324, to the cytosolic portion of cytochrome b_{558}. At this point p47-*phox* can be further phosphorylated. The kinetics of phosphorylation mirrors oxidase activation both in intact neutrophils (203) and in cell-free systems (37). Further analysis demonstrates that p47-*phox* may undergo a continuous cycle of phosphorylation and dephosphorylation throughout the period of superoxide release in PMA-stimulated neutrophils. The phosphorylation reaction predominates over dephosphorylation in the regulation of p47-*phox* status.

However, this pathway does not seem to be the only possible one. With receptor-mediated stimuli (81) and in the presence of staurosporine (phosphorylation inhibitor), translocation seems to occur independently from phosphorylation. In these conditions, p47-*phox* translocation appears to be linked to transmembrane signaling mechanisms involving perturbation in Ca^{2+} concentration and production of a second messenger from the hydrolysis of phospholipids. Furthermore, an additional observation showed that with appropiate stimuli, p47-*phox* translocation can occur even independently from Ca^{2+} concentration changes and from phosphorylation.

From all these observations it is possible to draw the following conclusions:

- p47-*phox* phosphorylation may be not the only modification required to promote translocation and activation of the system (184), (113);
- p47-*phox* phosphorylation occurs at different protein sites in different steps;
- a phosphorylation-dephosphorylation cycle may regulate the p47-*phox* status (77).

Independently from the modifications of p47-*phox* and p67-*phox* upon activation, translocation of the complex is an essential process for the activation of the NADPH oxidase (81). A continuous translocation is necessary to maintain the oxidase in an active state (81): the constant production of superoxide seems to be assured by the continuous recruitment of new units of NADPH oxidase, which remains only transiently in an activated state, due to the translocation of p47-*phox* and p67-*phox*.

Another fundamental aspect in p47-*phox*-p67-*phox* activities appears to be the binding to gp91-*phox*. This aspect was studied by the use of synthetic peptides corresponding to the C-terminus of gp91-*phox* (243). Synthetic peptides encompassing the gp91-*phox* region 559-565 were able to greatly impair both respiratory burst and p47-*phox* phosphorylation in fMet-Leu-Phe activated neutrophils. It was supposed that the peptides used were able to prevent the interaction between p47-*phox* and gp91-*phox* and to block the access of p47-*phox* to a specific kinase responsible for multiphosphorylation. Interestingly, the peptides were not able to prevent p67-*phox* binding to the membrane (165).

As far as p67-*phox* is concerned, it has been shown that its translocation follows that of p47-*phox* (140). The p67-*phox* translocation is completely dependent on the presence of p47-*phox* (112), while the contrary is not true. Less is known about p40-*phox*, except that it may bind

to the complex p47-*phox*/p67-*phox* and translocate towards the membrane upon cell activation. It is worth mentioning again that its presence is not required for activation of the oxidase in a cell-free assay.

4.5 NADPH oxidase regulation by Rac

The complexity of the regulation of the NADPH oxidase activation is further underlined by the discovery of a cytosolic GTP-binding factor that may be involved in the regulation of p47-*phox*/p67-*phox* translocation (218), (32). The factor has been identified as a complex of Rac and RhoGDP dissociation factor (GDI) (3), (2). Rac 1 is the protein isolated from guinea pig macrophages, while Rac 2 is the protein found in human neutrophils (143), (144).

Due to the fact that GTP analogs can modulate the translocation of p47-*phox*/p67-*phox* in a cell-free system (209), (286) and that no GTP-binding sites have been described in p47-*phox* and p67-*phox*, it was suggested a role for a GTP-binding protein in modulating the p47-*phox*/p67-*phox* interaction with the membrane components of NADPH oxidase. Rac is considered a good candidate as a regulatory element for the cytosolic components due to the following observations:

- it translocates from the cytoplasm to the plasma membrane of neutrophils upon cell activation (223);
- PMA and N-formyl peptide stimulation of cells cause a 5-20% translocation of the total amount of cytosolic Rac (similar amounts are described for both p47-*phox* and p67-*phox*) (30);
- translocation of the three proteins (Rac, p47-*phox* and p67-*phox*) occurs over a time that slightly precedes the kinetics of O_2^- production upon stimulation (figure 7);

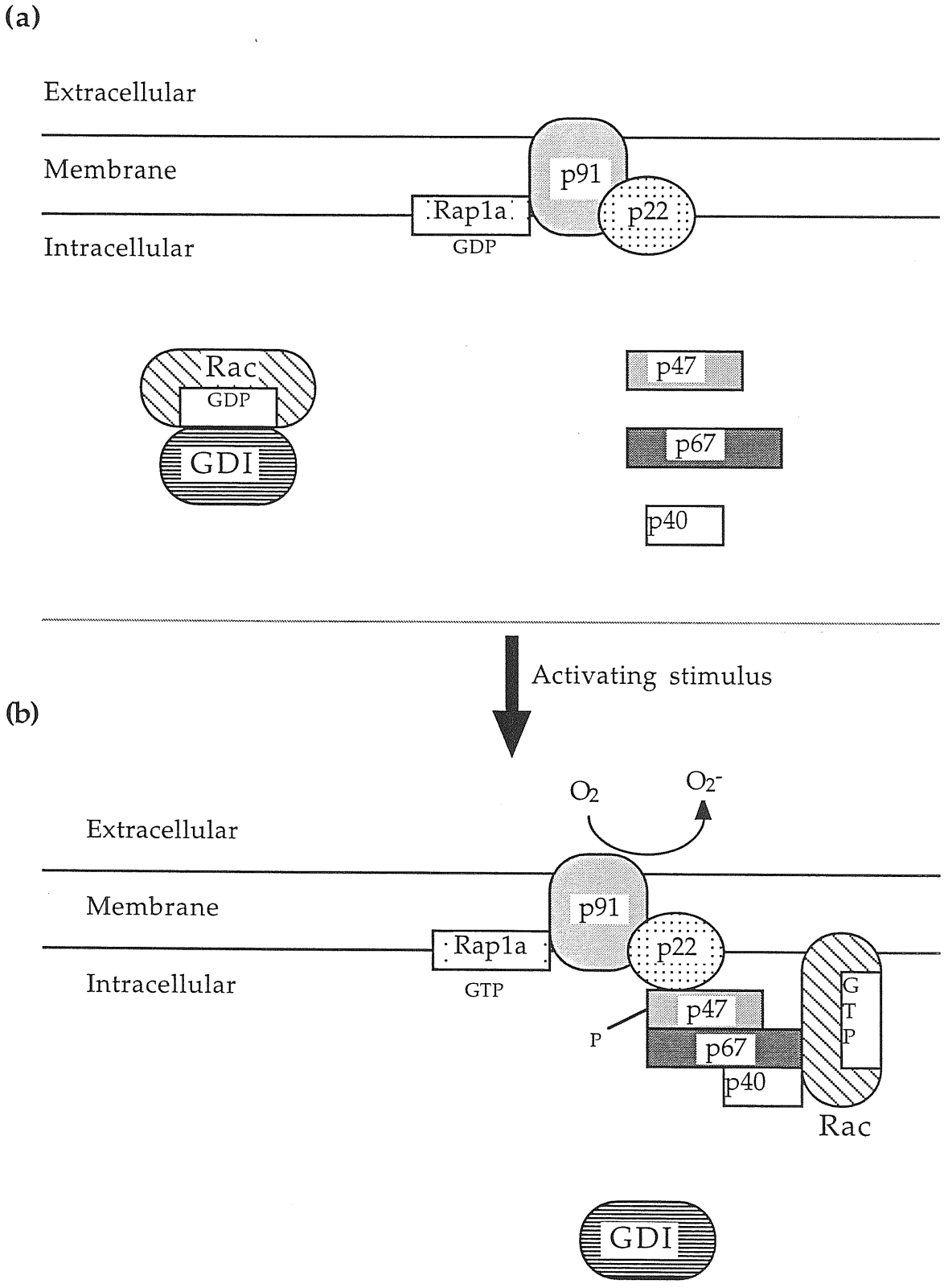


Figure 7. Schematic model of the human phagocyte NADPH oxidase in resting **(a)** and stimulated **(b)** cells. GDI: GDP dissociation inhibitor. P indicates phosphorylation of p47-*phox*

Modified from: Bokoch, G.M., 1994.

- very recently, it has been proposed (72) that p67-*phox* is the functional target for Rac in the phagocytic NADPH oxidase complex. Rac is able to bind to the NH_2-terminal 199 amino acids of p67-*phox*.

Regulation of Rac by RhoGDI represents a critical check point in the control of NADPH oxidase activation. Rac is active in the GTP-bound form (223): in this condition it is able to promote translocation. In the inactive form it binds to GDP and is linked with RhoGDI (135). The reactions allowing the modifications from the inactive to the active form are complex and not yet completely understood. A possible model is depicted in figure 8. GDI binds to Rac-GDP blocking its activities. In the presence of activating signals, a GDP dissociation stimulator (GDS) would be able to promote GDI dissociation from Rac (8), allowing Rac binding to GTP. GDS releases Rac-GTP leaving it in its active state. Terminating signals would be able to activate a GTPase activating proteins (GAPs) which is responsible to the Rac-GDP state (inactive).

The signals that are responsible for releasing Rac from RhoGDI remain to be defined. Recently, it has been proposed that several types of biologically active lipids, including arachidonic acid, phosphatidic acid and phosphatidylinositols (48), can disrupt binding of Rac to RhoGDI .

From all these data we can conclude that NADPH oxidase activity may be regulated at different steps and probably many redundant control systems are active at the same time.

4.6 Rap 1A

To complete the overview on the proteins involved in NADPH oxidase regulation, the so called GTP-binding protein Rap1A should also be mentioned. This protein has been described as a Ras-related protein

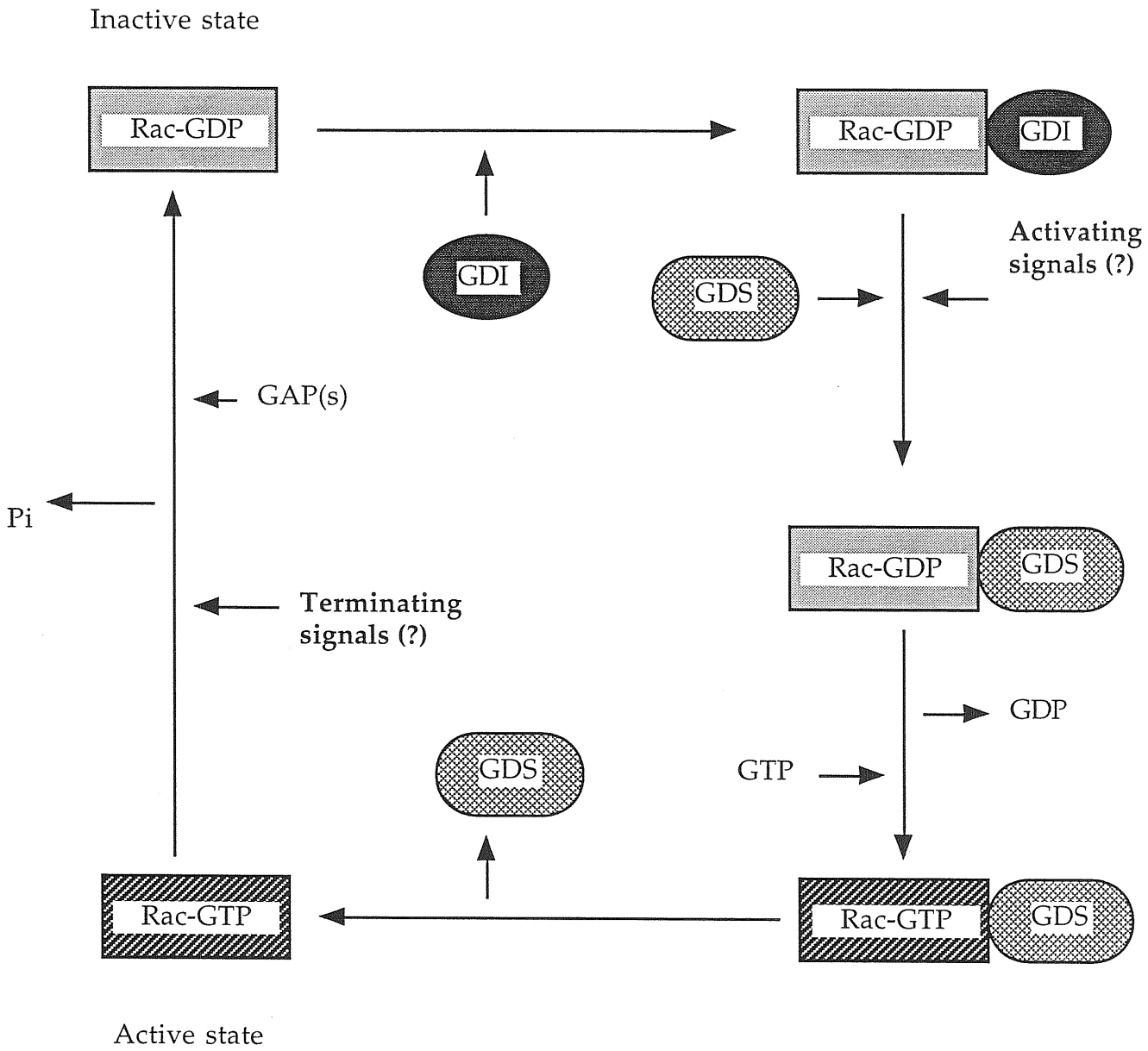


Figure 8. Diagrammatic representation of regulation of NADPH oxidase by Rac. GDI : GDP dissociation inhibitor. GDS: GDP dissociation stimulator.

Modified from: Bokoch, G.M., 1994.

(225) and it has been shown to be physically associated with the membrane components of NADPH oxidase since it can be co-isolated with cytochrome b by conventional purification procedures. Thanks to the fact that the p47-*phox*/p67-*phox* association can be modulated by GTP analogs (209), (286) and that neither of the proteins binds to GTP, a regulating role for Rap1A can be postulated, possibly in cooperation with the Rac protein.

5. The genetic basis of Chronic Granulomatous Disease

5.1 Gene location and tissue specificity

The genes for the two subunits of cytochrome b_{558}, p22-*phox* and gp91-*phox*, as well as those for p47-*phox* and p67-*phox*, have been localized, cloned, and characterized. Interestingly, the gene for gp91-*phox* was the first human gene to be identified by a positional cloning approach in 1989 (246). Table 4 summarizes these data.

The 8.5 kb gene for p22-*phox* is located on the long arm of chromosome 16 at 16q24 and contains six exons (75). The gene for the gp91-*phox* is located on the short arm of the X chromosome (Xq21.1) (74) and contains 13 exons spanning 30 kb (263). The p47-*phox* protein is encoded by a gene on the long arm of chromosome 7 at 7q11.23 (88), which contains 9 exons spanning 18 kilobases (41). The gene for the p67-*phox* is located on the long arm of chromosome 1 at position 1q25 (88). This gene spans 40 kb and contains 16 exons (134).

Interestingly, of the four "structural" components of the phagocytes NADPH oxidase, p22-*phox* is the only component expressed in cells other than phagocytes (212). However, although the mRNA is constitutively expressed, non-phagocytic cells contain little stable p22-*phox* protein. On the contrary, the other three structural proteins are expressed in a highly lineage-specific manner (193). Messenger RNAs for gp91-*phox*, p47-*phox* and p67-*phox* can be mostly detected in neutrophils, monocyte/macrophages, EBV-transformed B-cells (211), (121) and tonsilar B lymphocytes (167). In particular, the gp91-*phox* mRNA is abundant in neutrophils, perhaps accounting for 0.1 % or more of total cellular mRNA.

Still controversial is the presence (and the function) of NADPH oxidase components on the membranes of other cell types such as human

TABLE 4

		p22-*phox*	gp91-*phox*	p47-*phox*	p67-*phox*
Gene	Locus	CYBA	CYBB	NCF1	NCF2
	Chrom.location	16q24	Xp21.1	7q11.23	1q25
	Size	8.5 kb	30 kb	17-18 kb	40 kb
	Exons	6	13	9	16
mRNA	Size	0.8 kb	5 kb	1.4 kb	2.4 kb
Protein	Amino acids	195	570	390	526
	Mol.mass predicted	20.9 kDa	56 kDa	44.6 kDa	60.9 kDa
	Mol.mass SDS-PAGE	22 kDa	76-92 kDa	47 kDa	67 kDa
	pI	10.0	9.7	10	6
	Location in resting phagocyte	Membrane	Membrane	Cytoplasm	Cytoplasm
	Posttranslational modification	Phosphorylated	N-linked carbo-hydrates; Phosphorylated	Phosphorylated during oxidase activation	-

From: Roos, D., 1994.

fibroblasts (177), (178), and human mesangial cells or glomerular cells (227).

5.2 Classification of CGD

Defects in any of the four NADPH oxidase components lead to absence or reduction of enzymatic activity, and thus to the development of CGD. The complexity of the NADPH-oxidase system predicts that CGD has to be an heterogeneous disorder. Of the four major proteins identified as involved in the production of O_2^-, three are coded by autosomal genes, while one (gp91-*phox*) is coded by a gene located on the X chromosome. Therefore, the transmission of CGD occurs either as an X-linked or an autosomal recessive character.

a) X-linked transmission

Defects in the gene coding for gp91-*phox* account for all cases of X-linked CGD. This type of CGD is the most commonly encountered, accounting for 50-60% of all CGD patients. The different phenotypes are designated as $X91^0$ (50%) when no cytochrome b_{558} protein or heme is detectable, and as $X91^-$ (5-10%) or $X91^+$ (<5%) when subnormal or normal amounts respectively are detectable (235).

Alterations of the gene coding for gp91-*phox* found in affected individuals are represented by deletions, splice site mutations, missense mutations, nonsense mutations and insertions. All the mutations so far described in the literature are reported in table 5 (and schematically in figure 9). It should be considered that 47 different mutations have been found in 49 families. This indicates that several sites of the protein are extremely sensitive to mutations.

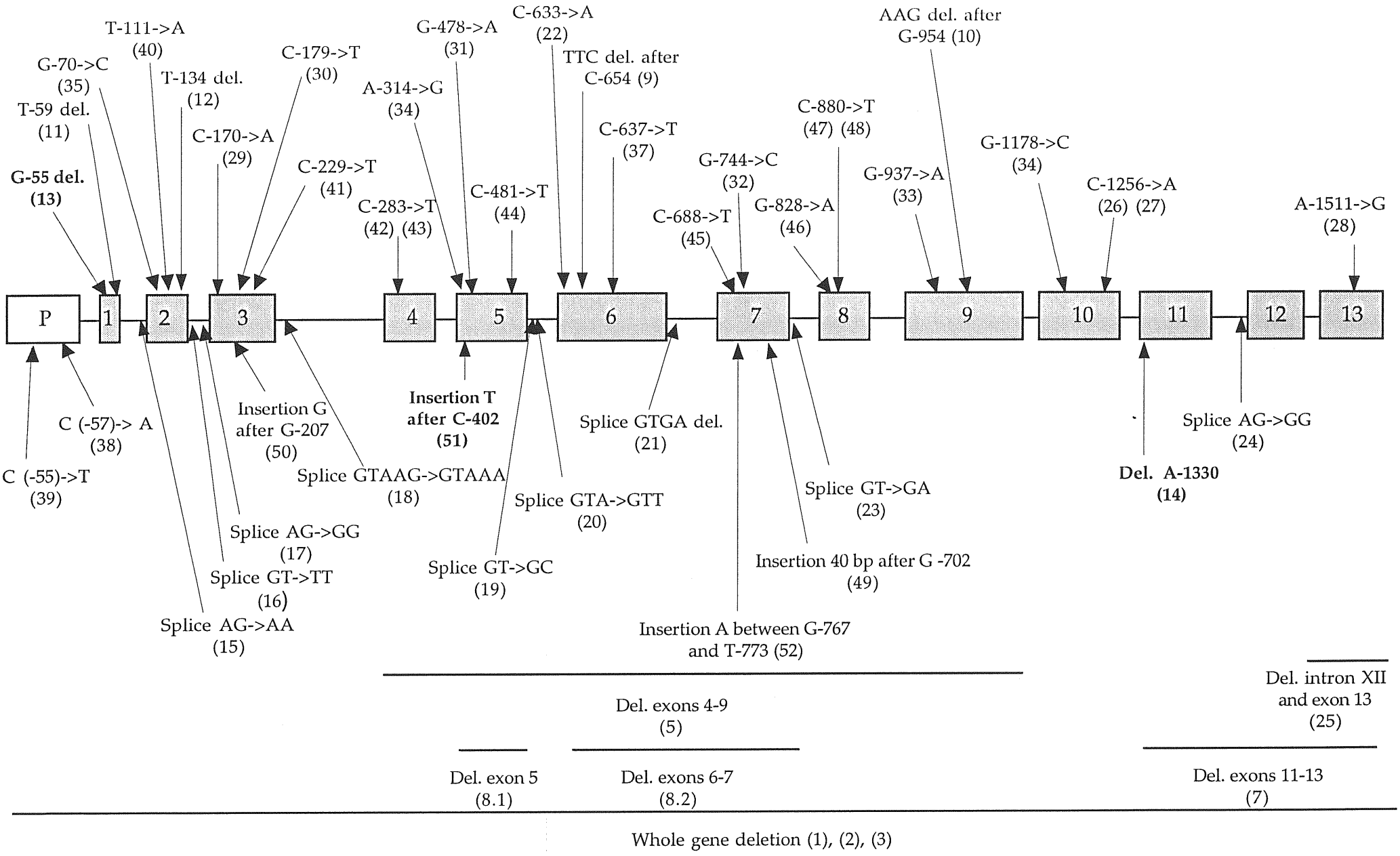


Figure 9 (legend on following page)

Figure 9.

All the known defects detected so far in the gp91-*phox* gene are shown. Exons are represented by rectangles, introns by lines.
Large deletions are indicated by lines; all the other defects are indicated by arrows. Bold typed arrows indicate the three new defects described in this thesis.
The length of introns is not proportional to that of exons. Numbering in parenthesis is referred to the progressive numbering of patients reported in table 5

TABLE 5

Nr.	Patient	Sex	CGD type	Mutation type	NADPH oxidase activity	Cytochrome b_{558} protein	Cytochrome b_{558} spectrum	mRNA gp91-*phox*	Nucleotide change	Amino acid change	Reference
1.	B.B.	M	X91°	deletion	(0)	(0)	(0)	(0)	~5000 kb deletion	N.A.	Francke et al. 1985
2.	N.F.	M	X91°	deletion	(0)	(0)	(0)	(0)	~4000 kb deletion	N.A.	Royer-Pokora et al. 1986
3.	O.M.	M	X91°	deletion	0	0	0	0	~800 kb deletion	N.A.	Frey et al. 1988
4.	S.B.	M	X91°	deletion	0	(0)	0	N.D.	N.D.	N.D.	de Saint-Basile et al. 1988
5.	T.S.	M	X91°	deletion	N.D.	N.D.	N.D	N.D.	~14 kb deletion	deletion of exons 4-9, frameshift	Roos 1993
6.	P.T.	M	X91°	deletion	(0)	(0)	0	0	~10 kb deletion	N.R.	Pelham et al. 1990
7.	M.H.	M	X91°	deletion	0	0	0	decreased	at least 6.5 kb deletion from exon 11-3' UT	deletion of exons 11-13	
8.1	T.W.	M	X91°	deletion	0	0	0	decreased	~3 kb deletion	deletion of exon 5	Roos 1993
8.2	N.W.	M	X91°	deletion	0	0	0	decreased	~3.5 kb deletion	deletion of exon 6+7	Roos 1993

From: Roos, D., 1994

TABLE 5
Continued

Nr.	Patient	Sex	CGD type	Mutation type	Cytochrome b_{558} NADPH oxidase activity	Cytochrome b_{558} protein	Cytochrome b_{558} spectrum	mRNA gp91-*phox*	Nucleotide change	Amino acid change	Reference
9.	C.G.	M	X91°	deletion	0	0	0	N.D.	TTC deletion after C-654	in-frame deletion of Phe-215 or Phe-216	
10.	-	M	X91-	deletion	~24%	~21%	N.R.	N	AAG deletion after G-954	in-frame deletion of Lys-315	Curnutte 1993
11.	T.F.	M	X91°	deletion	0	0	0	N.D.	T-59 deletion	frameshift, stop in codon 21	Roos 1993
12.	G.Q.	M	X91°	deletion	0	0	0	N	T-134 deletion	frameshift, stop in codon 60	Roos 1993
13.	N.B.	M	X91°	deletion	0	0	N.D.	N	G-55 deletion	frameshift, stop in codon 21	Zentilin et al. 1994
14.	A.Z	M	X91°	deletion	0	0	N.D.	decreased	A-1330 deletion	frameshift, stop in codon 501	Zentilin et al. 1994
15.	-	M	X91°	splice/deletion	0	0	0	N.D.	splice AG ->AA at end of intron I	deletion exon 2 (in frame)	De Boer et al. 1992
16.	-	M	X91°	splice/deletion	0	0	N.R.	N	splice GT ->TT at start of intron II	deletion exon 2 (in frame)	Curnutte 1993

TABLE 5
Continued

Nr.	Patient	Sex	CGD type	Mutation type	NADPH oxidase activity	Cytochrome b_{558} protein	Cytochrome b_{558} spectrum	mRNA gp91-*phox*	Nucleotide change	Amino acid change	Reference
17.	-	M	X91°	splice/deletion	0	0	0	N.D.	splice AG ->GG at end of intron II	deletion exon 3 (in frame)	Curnutte et al. 1993
18.	R.W.	M	X91°	splice/deletion	0	0	0	decreased	splice GTAAG ->GTAAA at start of intron III	deletion exon 3 (in frame)	De Boer et al. 1992
19.	-	M	X91°	splice/deletion	(0)	(0)	(0)	N.D.	splice GT ->GC at start of intron V	deletion exon 5,frame-shift,stop in codon 133	Curnutte et al. 1993
20.	D.D.	M	X91°	splice/deletion	0	0	0	decreased smaller	splice GTA->GTT at start of intron V	deletion exon 5,frame-shift,stop in codon 133	De Boer et al. 1992
21.	B.S.	M	X91-	splice/deletion	0	N.D.	~10%	N.D.	splice GTGA deletion at start of intron VI	deletion exon 6,frame-shift	
22.	R.H.	M	X91°	splice/deletion	0	0	0	N.D.	C-633->A	partial deletion exon 6, frameshift, stop in codon 206	De Boer et al. 1992

TABLE 5

Continued

Nr.	Patient	Sex	CGD type	Mutation type	Cytochrome b_{558} NADPH oxidase activity	protein	spectrum	mRNA gp91-*phox*	Nucleotide change	Amino acid change	Reference
23.	C.B.	M	X91°	splice/deletion	0	0	0	decreased	splice GT ->GA at start of intron VII	deletion exon 7,frame-shift,stop in codon 230	De Boer et al. 1992
24.	M.G.	M	X91-	splice/deletion	6%	N	N	N	splice AG ->GG at end of intron XI	deletion aa 488-497 in exon 12 (inframe)	Schapiro et al.1991
25.	J.W.	M	X91°	splice/deletion (?)	0	0	0	0	~1 kb deletion from intron XII to 3' UT	deletion C-terminal 41 aa (exon13)	Royer-Pokora et al. 1986
26.	R.C./ D.C.	2M	X91+	missense	0	N	N	N	C-1256->A	Pro-415->His	Dinauer et al. 1989
27.	D.R.	M	X91+	missense	0	N.D.	N	N.D.	C-1256->A	Pro-415->His	
28.	D.S.	M	X91+	missense	0	N	N	N	A-1511->G	Asp-500->Gly	Leusen et al. 1994
29.	O.G.	M	X91-	missense	0	N.D.	~30%	N.D.	C-170->A	Ala-53->Asp	
30.	H.K.R. J.K.R.	2M	X91-	missense	20-25 %	decrea sed	~60%	N	C-179->T	Pro-56->Leu	
31.	R.L.	M	X91-	missense	~5%	decrea-sed Mr increa-sed	~8%	N	G-478->A	Ala-156->Thr	Bolscher et al. 1991
32.	J.L.	M	X91-	missense	5-10%	0	~40%	N	G-744->C	Cys-244->Ser	Bolscher et al. 1991

TABLE 5

Continued

Nr.	Patient	Sex	CGD type	Mutation type	Cytochrome b_{558} NADPH oxidase activity	protein	spectrum	mRNA gp91-*phox*	Nucleotide change	Amino acid change	Reference
33.	D.H. T.C.	2M	X91-	missense	3-9%	<10%	10-15%	N	G-937->A	Glu-309->Lys	Curnutte et al. 1993
34.	F.B.	M	X91-	missense	10-20%	decreased Mr increased	~20%	N	G-1178->C	Gly-389->Ala	Bolscher et al. 1991
35.	-	M	X91°	missense	0	0	N.R.	N.D.	G-70->C	Gly-20->Arg	Curnutte et al. 1993
36.	E.P.	F	X91°	missense	(0)	(0)	(0)	N	A-314->G (heterozygous)	His-101->Arg	Bolscher et al. 1991
37.	P.B.	M	X91°	missense	0	0	0	N	C-637->T	His-209->Tyr	Bolscher et al. 1991
38.	-	M	-	missense	-	reduced	N.D.	reduced	C(-57)->A in the promoter region	-	Newburger et al. 1994
39.	-	M	-	missense	-	reduced	N.D.	reduced	C(-55)->T in the promoter region	-	Newburger et al. 1994
40.	M.Z.	M	X91°	nonsense	0	0	0	N	T-111->A	Tyr-33->stop	
41.	B.C.	M	X91°	nonsense	0	0	0	N	C-229->T	Arg-73->stop	Bolscher et al.1991
42.	-	M	X91°	nonsense	0	0	0	N.D.	C-283->T	Arg-91->stop	
43.	W.L.	M	X91°	nonsense	0	0	0	N.D.	C-283->T	Arg-91->stop	Curnutte et al. 1993
44.	-	M	X91°	nonsense	0	0	0	N.D.	C-481->T	Arg-157->stop	Curnutte et al. 1993

TABLE 5
Continued

Nr.	Patient	Sex	CGD type	Mutation type	NADPH oxidase activity	Cytochrome b_{558} protein	Cytochrome b_{558} spectrum	mRNA gp91-*phox*	Nucleotide change	Amino acid change	Reference
45.	-	F	X91°	nonsense	(0)	(0)	N.R.	(0)	C-688->T (heterozy-gous)	Arg-226->stop	Curnutte 1993
46.	R.R.	M	X91°	nonsense	0	N.D.	0	N.D.	G-828->A	Trp-272->stop	
47.	-	M	X91°	nonsense	0	0	0	N.D.	C-880->T	Arg-290->stop	Curnutte et al. 1993
48.	J.M.	M	X91°	nonsense	0	0	0	N.D.	C-880->T	Arg-290->stop	Curnutte et al. 1993
49.	P.E.	M	X91°	insertion	0	0	0	decreased	insert 40 bp after G-702 in exon 7	13 additional aa after Gly-230,frame-shift,stop in codon 253 (exon 7)	Rabbani et al. 1993
50.	-	M	X91°	insertion	0	0	0	low	insert G after G-207 in exon 3	frameshift, stop in exon 4	Curnutte et al. 1993
51.	A.G.	M	X91°	insertion	0	0	N.D.	N	insert T after C-402	frameshift, stop in codon 133	Zentilin et al. 1994
52.	-	M	X91°	insertion	0	0	N.R.	0	insert A between G-767 and T-773	frameshift, stop in exon 8	Curnutte 1993

0, zero; (0) presumed to be zero, judging from the mutation; N.A. not applicable; N.D. not determined; N.R., not reported; 3' UT 3' untranslated mRNA region; Patients 8.1 and 8.2 are brothers, patients 26 are two brothers, patients 30 are also two brothers, and patients 33 are maternal first cousins. Patients 36 and 45 are female patients with extreme lyonization; in these patients the control allele was found as well. Data are obtained from Roos, D. et all. with modifications.

Deletions

Any kind of deletion can be found in affected patients as it is possible to deduce from table 5 (patients 1-14) (89), (246), (90), (68), (234), (215), (62), (320). With the exception one case (patient 10), deletions lead to a X91^{o} subtype of CGD. Large deletions involving the entire gene for gp91-*phox* and neighboring genes have been described in three patients (patients 1-3). In these cases other clinical syndromes are present at the same time in affected individuals. The most common associations are represented by Duchenne muscular dystrophy, retinitis pigmentosa and McLeods's syndrome (147), (89), (90), (68). Partial deletions of the gene have been detected in four patients with loss of different exons (patients 5-8). Triplet base pair deletions that predict in frame deletion of one aminoacid have been detected in patients 9 and 10. Interestingly, patient 10 shows a residual NADPH activity. This probably means that the in-frame deletion (absence of Lys-315) affects only the stability and not the function of the gp91-*phox* protein. Patients 11-12 are characterized by single base pair deletions. In both cases a premature termination of protein translation occurs. The last two patients ,13 and 14, will be described in this thesis.

Splice-site mutations

Eleven of the patients so far described (65), (62), (59), (251), (246) carry splice site mutations. In general, splice sites mutation leads to severe form of the disease (235). In patients 16, 18, 19, 20, 21 and 23 a single nucleotide substitution in the donor splice sites of the relevant introns is responsible for exon skipping during mRNA processing. In patients 15 and 17 the

acceptor splice sites of introns I and II respectively, carries a missense mutation. The consequence is the exon skipping during mRNA processing. A similar mutation was detected in patient 24. In this case, the mutation lies in the acceptor splice site of intron XI. The partial skipping of exon 12 could be explained by the activation of a cryptic splice site in exon 12. Only 30 nucleotides are lost during mRNA processing with the consequence of an in-frame deletion of 10 amino acids in the COOH-terminus of the gp91-*phox* protein. According to the residual oxidase activity of his neutrophils (about 6%), this patients has to be considered as an $X91^-$ subtype of CGD. An opposite situation occurs in patients 22. In this case a mutation in exons 6 seems to create a new splice site that is preferred over the normal donor splice site of intron VI. The consequence is the skipping of exon 6 from the mutation to the 3' end of the exon. In addition, the mutation induces the formation of a frameshift and a premature stop codon. Finally, in patients 25 a deletion of exon 13 is probably due to a mutation in the acceptor splice site of intron XII. The instability of mRNA can be explained by the absence of exon 13 that contains the 3' untranslated region of the gp91-*phox* mRNA.

Missense mutations

In the cases studied so far (73), (159), (31), (59), single amino-acid replacements have no effect on mRNA stability but lead to either $X91^+$, $X91^-$ or $X91^o$ CGD. Among four patients (patients 26-28) with $X91^+$ CGD three carry a mutation that leads to Pro 415-His substitution. The substitution has no effect on the stability of gp91-*phox* as it can be detected in western blotting but it prevents NADPH binding (256). The other patient has an Asp 500 - Gly substitution that does not affect protein stability but prevents NADPH - oxidase activation by p47-*phox* and/or p67-

phox (159). Eight patients (patients 29-34) with X91$^-$ CGD have been described. In general, the mutations are located in the middle portion of the protein. Mutations seem to decrease the stability of the protein or its association with the p22-*phox* subunit. Only three patients (patients 35-37) with missense mutations leading to complete absence of the protein have been described. Mutations are either in the N-terminal half of the protein which contains most of the hydrophobic stretches that might serve as membrane-spanning regions, or remove histidyl residues that might be involved in heme binding.

Nonsense mutations

Nine patients (40-48) carrying nonsense mutations have been described (31), (59), (62). Interestingly, seven of them have C->T substitutions, changing the CGA codon for Arg into the TGA stop codon. All those mutations lead to X91^o phenotype of CGD.

Insertions

The last type of mutations found in CGD patients are represented by insertions (226), (59), (320). Two patients (50,52) carry single nucleotide insertions that are responsible for a frameshift that predicts a premature termination of gp91-*phox* protein synthesis. The CGD subtype is obviously represented by X91^o form. Another patients (49) was found to have a 40-base-pair insertion at intron VI/exon 7 boundary. This proved to be a 40-bp repeat, probably due to unequal crossing-over. The consequence is the presence of 13 additional aminoacids and a premature termination of gp91-*phox* synthesis due to a frame shift. In all three cases the mRNA stability is greatly decreased. The clinically severe X91^o CGD subtype can be

explained by the presence of a truncated form of the protein gp91-*phox*. Patient 51 will be described in this thesis.

Mutations in the promoter region

Very recently (197), two point mutations (A-> C-57 and T->C-55) have been identified in the gp91-*phox* promoter region of two different children (patients 38-39). These two mutations are responsible for a diminished expression of immunoreactive gp91-*phox* protein and mRNA, with relative preservation of transcription initiation at an alternative start site at nucleotide 190 of the normal cDNA sequence. Each mutation abolishes the association of oligonucleotides corresponding to the gp91-*phox* promoter region with a DNA-binding protein detectable on gel shift assay.

b) Autosomal recessive transmission.

Mutations in p22-*phox*

Mutations that inactivate p22-*phox* lead to an autosomal form of CGD (75), (64), (117), (76). Only the subjects homozygous for the defect are sick. This type of CGD is rare, probably accounting for less than 10% of all CGD patients. The different phenotypes are designed as $A22^{+}$ (<1%), $A22^{o}$ (5-10%) and $A22^{-}$, when cytochrome b_{558} protein or heme are present in normal amounts, undetectable, or diminished respectively. Table 6 shows all the patients so far reported. As well as for gp91-*phox*, a great number of defects have been described also for p22-*phox*.

TABLE 6

Nr.	Patient	Sex	CGD type	Mutation type	NADPH oxidase activity	Cytochrome b_{558} protein	Cytochrome b_{558} spectrum	mRNA p22-*phox*	Nucleotide change	Amino acid change	Reference
1	L.N.	F	A22°	deletion (homozygous)	0	0	0	N	>10 kb deletion	N.A.	Dinauer et al. 1990
2	G.S.	M	A22°	1) deletion 2) missense	0	0	0	N	1)C-272 deletion 2)G-297->A	1)frameshift 2)Arg-90->Gln	Dinauer et al. 1990
3	O.P.	F	A22°	missense (homozygous)	0	0	0	N	C-382->A	Ser-118->Arg	Dinauer et al. 1990
4	fam.S.	2F 1M	A22°	missense (homozygous)	0	0	0	N	G-297->A	Arg-90->Gln	De Boer et al. 1992
5	A.G.	F	A22°	missense (homozygous)	0	0	0	N	A-309->G	His-94->Arg	De Boer et al. 1992
6	S.B.	M	A22°	1) missense 2) insertion	0	0	0	N	1)A-186->G 2)insert G between C-194 and A-200	1)Glu-53->Val 2)frameshift, chain elongation, stop at codon 211	Hossle et al. 1994
7	W.d.S.	M	A22°	splice/deletion (homozygous)	0	0	0	N	splice GTGA ->ATGA at start of intron IV	deletion exon 4	De Boer et al. 1992
8	I.L.	F	A22+	missense	0	N	N	N	C-495->A	Pro-156->Gln	Dinauer et al. 1991

From: Roos, D., 1994

A big deletion was found only in one patient (patient 1) while point mutations were found in other five subjects (patients 2-6). These point mutations were all found in the open reading frame. Patients 2 and 6 carry also a deletion and an insertion respectively. They are compound heterozygotes for two mutations that predict frameshifts and a non conservative aminoacid replacements. Patient 7 carries a point mutation in the consensus donor splice site sequence of the flanking intron sequence of exon 4. The defect in mRNA splicing leads to skipping exon 4. Due to the fact that this is an in-frame deletion, a shortened polypeptide is predicted to be synthesized. Patient 8 has the $A22^{+}$ form of CGD. His cytochrome b_{558} is present but not active. This is due to a Pro156->Gln substitution. Due to the fact that the substitution occurs in the p22-*phox* cytoplasmatic domain it is possible to argue that an interference in cytochrome b_{558} p47-*phox* interaction occurs.

Mutations in p47-*phox*

Mutations in the p47-*phox* gene always lead to complete absence of the protein, and thus to the $A47^{o}$ CGD phenotype. Patients with this subtype of CGD comprise about 30% of all CGD patients.

Only four different genetic defects have been reported so far to cause A47 CGD. They are represented by: a) GT deletion in a tandem repeat GTGT corresponding to the first four bases of exon 2 (39), (41), (295), (123); b) two point mutations: A179->G and A425->G; c) deletion of G502. Patients homozygous for the GT deletion present a frameshift and premature translation termination after the synthesis of a 50-amino-acid protein. There are also patients compound heterozygotes for the GT deletion in combination with the point mutations or the deletion described above.

Although the mRNA is present in apparently normal amounts and with normal size (162), (39), (41) in all patients tested, p47-*phox* is always undetectable in neutrophil lysates. This probably means that these mutations lead to the synthesis of an unstable form of the protein. The great number of polymorphisms described indicate that p47-*phox* is less dependent on a critical conformation for its function than the cytochrome b_{558} subunits.

Mutations in p67-*phox*

Only A67^{o} CGD patients are known. This CGD subtype is rare, accounting for less than 5% of all CGD patients. The heterogeneity of defects in p67-*phox* seem to be larger than that in p47-*phox*. Three different defects have been described in three different subjects. One patient is homozygous for a G233->A substitution while the other two are homozygous for two different deletions. The first deletion is represented by exon 3 deletion probably due to a splice sites mutation (235). The second deletion is a GAA deletion predicting a Lys-58 deletion (235). All the patients tested have normal amounts of mRNA for p67-*phox* but no protein (158), (66).

6. B-cell lines as an *in vitro* model of CGD

Permanent lymphoblastoid B cell lines are of great practical value in human clinical and experimental genetics. They can be simply obtained by transformation of peripheral B lymphocytes by Epstein-Barr virus (EBV) (194). Compared to other methods of long-term cultivation (i.e. tissue culture of skin fibroblasts) these cell have a number of advantages:

- lymphocytes can be easily obtained from any patient;
- EBV-transformed lines exhibit chromosomal stability up to high passages;
- they are the ideal source for molecular studies in humans as repeated DNA, RNA and cellular protein preparation can be obtained without great effort; in particular they represent a good source of biological patient samples for all those cases where it is not possible to obtain repeated and/or abundant blood samples.
- they grow in suspension and their minimal pretension concerning medium allows the cultivation up to high cell density without much expenditure of works.

In addition to all these general advantages, EBV immortalized B cells have some features that make them a good model for studying CGD. Actually, they have some characteristics in common with differentiated granulocytes, for example the enhanced expression of surface adhesion molecules such as CD2, CD48, and LFA-3 as well as expression of the granulocyte-specific *fgr* oncogene (100), (213). However, as far as the study of CGD is concerned, the most relevant feature is represented by the acquisition of the ability to generate superoxide after 4-12 weeks from immortalization (295). B-cell lines show the presence of a certain amount of functional cytochrome b_{558} at their membrane (294). In particular, it has

been demonstrated that cytochrome b_{558} is present at a concentration 10 times lower than in neutrophils (55), and that the superoxide generation rate in these cells is also 10 times lower (294). Despite this diminished levels of oxidase production, O_2^- production can be easily detected and is strictly dependent on specific stimulation. The function of superoxide production, if any, is unknown.

Stimuli that are able to induce superoxide generation in EBV transformed B cell lines include phorbol myristate acetate, calcium ionophores, and surface immunoglobulin cross-linking agents (166), (103), (155). As in PMNs, the B cell lines enzyme is not inhibited by cyanide but it is higly sensitive to diphenylene iodium and iodium biphenyl (58), (318).

All these data demonstrate that B-cell lines posses, although at a lower level, the same functional NADPH oxidase as that found in phagocytic cells. Furthermore, it has been demonstrated that B-cell lines from CGD patients show the same oxidase dysfunction as that found in phagocytes from the same patients (294), (220). For these reasons, EBV immortalized B lymphocytes from CGD patients not only are a useful tool for studying the genetic defects leading to the disease, but can also be used as an in vitro model of the disease for monitoring the expression and function of the gp91-*phox* after gene transfer.

7. Gene therapy for CGD

Gene replacement therapy has been suggested for many single gene disorder using viral vectors or expression vectors. However, only some of the single gene disorders so far described can benefit from gene therapy. The main features required for a potential successful gene therapy approach to a genetic disease are the following:

1) the genetic defect has to be known and the target gene has to be cloned;
2) the ideal target cell should be able to renew itself and differentiate into progeny cells after transplantation to generate a sizable self-perpetuating cells mass that contains the transferred gene for the entire life span of the patient;
3) a partial reconstitution of the genetic defect should result in a clinical improvement or cure;
4) the genetic disorder has high morbidity and mortality rates and the conventional symptomatic treatment has limited success.

CGD possesses all these requirements. It is a disorder of marrow-derived cells with well-defined genetic defects and less than 10% of normal cells may be responsible for a normal phenotype (236). Additionally, bone marrow transplantation as therapeutic approach for the disease has, at best, sporadic success (129). For all these reasons CGD can be considered a good candidate for gene therapy.

Early attempt in CGD gene therapy concerned the correction of genetic defect in B-cell lines from affected patients. Although these cells do not represent the final target of gene therapy, nevertheless they are a good tool for studying the reconstitution of the genetic defect as detailed in the preceding paragraph. Actually, recent studies from several laboratories

have demonstrated that p47-*phox* protein expression and NADPH oxidase activity can be partially restored in EBV-transformed B-lymphocytes lines established from A47° CGD patients after transduction or transfection with retrovirus or other expression vectors containing p47-*phox* cDNA (50), (281), (295), (42). In addition, transfection of EBV B-cell lines from X91° CGD patients with a vector containing gp91-*phox* cDNA has been reported to partially correct gp91-*phox* protein expression and NADPH oxidase activity (221). Analogous results have been obtained by transfection with an expression plasmid containing a p22-*phox* cDNA of B-cell lines from two A22° CGD patients (168).

However, EBV-transformed lymphocytes are not relevant targets for gene therapy of CGD, since these cells are different from the myelomonocytic cells that are deficient in CGD. An important step, therefore, was the publication (258) of a work concerning the successful transduction of peripheral blood hematopoietic progenitors with a retroviral vector bearing p47-*phox* cDNA. The procedure described resulted in partial correction of NADPH oxidase activity when the hematopoietic progenitors were differentiated *in vitro* to mature neutrophils and monocytes. Another recently published work (161), showed the possibility to transduce $CD34^+$ hematopoietic progenitors cells with retroviral vectors containing gp91-*phox* or p22-*phox* cDNA. The partial correction of the genetic defect was demonstrated in mature cells after *in vitro* differentiation.

RESULTS

The experimental results described in these thesis were obtained from samples of three patients (A.G., A.Z. and N.B.) affected by CGD. A.G. and A.Z. are followed at the Children Hospital Burlo Garofalo in Trieste. Both the patients are males (15 and 21 years old respectively) and are affected by the X-linked form of the disease. They have a long history of recurrent infections badly controlled by anti microbial therapy. A brother of patient A.G., also affected by CGD, died in 1991 after a long history of disease. More recently, samples from another patient (N.B.) were obtained. This patient is 9 years old and is also affected by X-CGD, but his disease is milder. He is followed at the Ospedale Generale Regionale Miulli in Bari.

Due to the clear X-linked transmission of the disease, resulting both from clinical data and from the results of the NBT test on other members of the family, attention was concentrated on the gene encoding for the gp91-*phox* subunit of cytochrome b_{558}, which is located on the X chromosome.

CHARACTERIZATION OF THE GENETIC DEFECT

In order to clearly characterize the genetic defect responsible for the disease in these three patients a set of assays was performed. To exclude the possibility that large deletions were responsible for the disease, the DNA of all three patients was analyzed by southern blotting assay. As a source of DNA, the lymphoblastoid cell lines obtained by EBV immortalization of blood mononuclear cells form each patient were used. The second step in DNA analysis was the complete sequencing of the gp91-

phox gene coding region. To investigate the gp91-*phox* gene expression a very accurate, sensitive and reproducible method for quantitation of low abundance mRNAs technique was set up as well as a northern blotting assay in order to check the gp91-*phox* mRNA size. The cytochrome b_{558} presence was then investigated by using antibodies against the gp91-*phox* protein and p22-*phox* protein. Finally we studied the superoxide generation (luminol chemiluminescence test) by lymphoblastoid cell lines obtained from the three patients and compared the results with those of normal controls.

1. Establishment of lymphoblastoid cell lines

Lymphoblastoid cell lines were established for healthy controls and for the three patients analyzed in this study.

Normal B-cell lines express a low amount of functional cytochrome b_{558} on their membrane (294). It has also been demonstrated that B-cell lines from CGD patients show the same oxidase disfunctions as that found in the phagocytes from the same patients (294), (220). For these reasons they can be utilized to study in details several biological aspects concerning the genetics and the functionality of the NADPH oxidase subunits. Furthermore, since the amount of blood that can be drawn from these children is limited, B cell lines offer an unlimited source of pathological samples.

In this work B cell lines were used as source of cellular membranes, genomic DNA, and messenger RNA. Lymphoblastoid cell lines are also very important in the development of strategies for gene therapy since they constitute a very useful tool for monitoring gene expression after transduction of expression vectors bearing the gene of interest.

As source of immortalizing virus, the supernatant of the lymphoblastoid marmoset cell line B95-8 containing high titers of infectious Epstein Barr Virus (EBV) was used. The supernatant was added to purified mononuclear cells of both patients and normal controls. Mononuclear cells were separated by density gradient centrifugation from ten milliliters of blood samples drawn at most 12 hours before. Flasks containing infected cells were then incubated upright at 37°C. Twenty four hours later, half of the virus-containing medium was replaced by fresh medium. Cyclosporin A was than added to the cultures. This drug is able to avoid activation of reactive T lymphocytes clones previously sensitized against EBV antigens (the serological reactivities to EBV of the three patients and of the normal controls was not known). Cyclosporin A addition is absolutely required within the first 24 hours after infection with EBV since it acts only during the T cell activation. Once activated and functionally mature, T cells cannot be inactivated by the drug (207), (282). In previously immunized patients, activation of T lymphocytes represents the main cause of lymphoblastoid cell line regression.

Two distinct morphologic features can be observed few days after infection. First, blastogenesis becomes evident resulting in enlargement of the lymphocytes and, second, there is increasing development of cell aggregates of proliferative lymphoblastoid cells. EBV-infected cells increase in number during the following weeks. Each cell culture was subsequently expanded and frozen as immortalized stocks of cells derived from patients and normal controls.

2. Genomic DNA analysis

2.1 Southern blotting

In order to evaluate the presence of gross deletions within the gp91-*phox* gene, Southern blot analysis was performed with patients' DNA (A.G., A.Z. and N.B.) extracted from established lymphoblastoid B cell lines. A DNA fragment containing a portion of the gp91-*phox* cDNA was used as probe for hybridization. The results of the hybridization are shown in figure 10. DNA samples from the three patients and two samples from normal individuals were digested with *Hind III*, *Nsi I* and *Eco RI* (in this last case four normal controls were digested), the resulting fragments resolved by gel electrophoresis, transferred to a nylon membrane and hybridized. It is apparent from figure 10 that the hybridization pattern for *Hind III* and *Eco RI* enzymes is indistinguishable for either the patients and the normal individuals. The hybridization pattern for *Nsi I* detects two sets of polymorfic DNA fragments in a normal control (N2) and in patient A.G. (of 2,9 or 2,5 kb and 1,7 or 1,3 kb respectively) (215).

As stated above, only a part of the cDNA (1.9 kb) was available to us as hybridization probe. As a consequence, only a portion of the gp91-*phox* gene, totally spanning about 30 kb (266), was explored by this hybridization. Therefore, deletions in intronic regions not revealed by the probe could not be detected by this analysis. Nevertheless, gross deletions of the genomic locus or selective deletions of the coding sequence of the gene are excluded in these patients. These data are not surprising if we think that only 19% of the patients so far described, carry big deletions detectable by southern blotting analysis (89), (246), (90), (68), (234), (215).

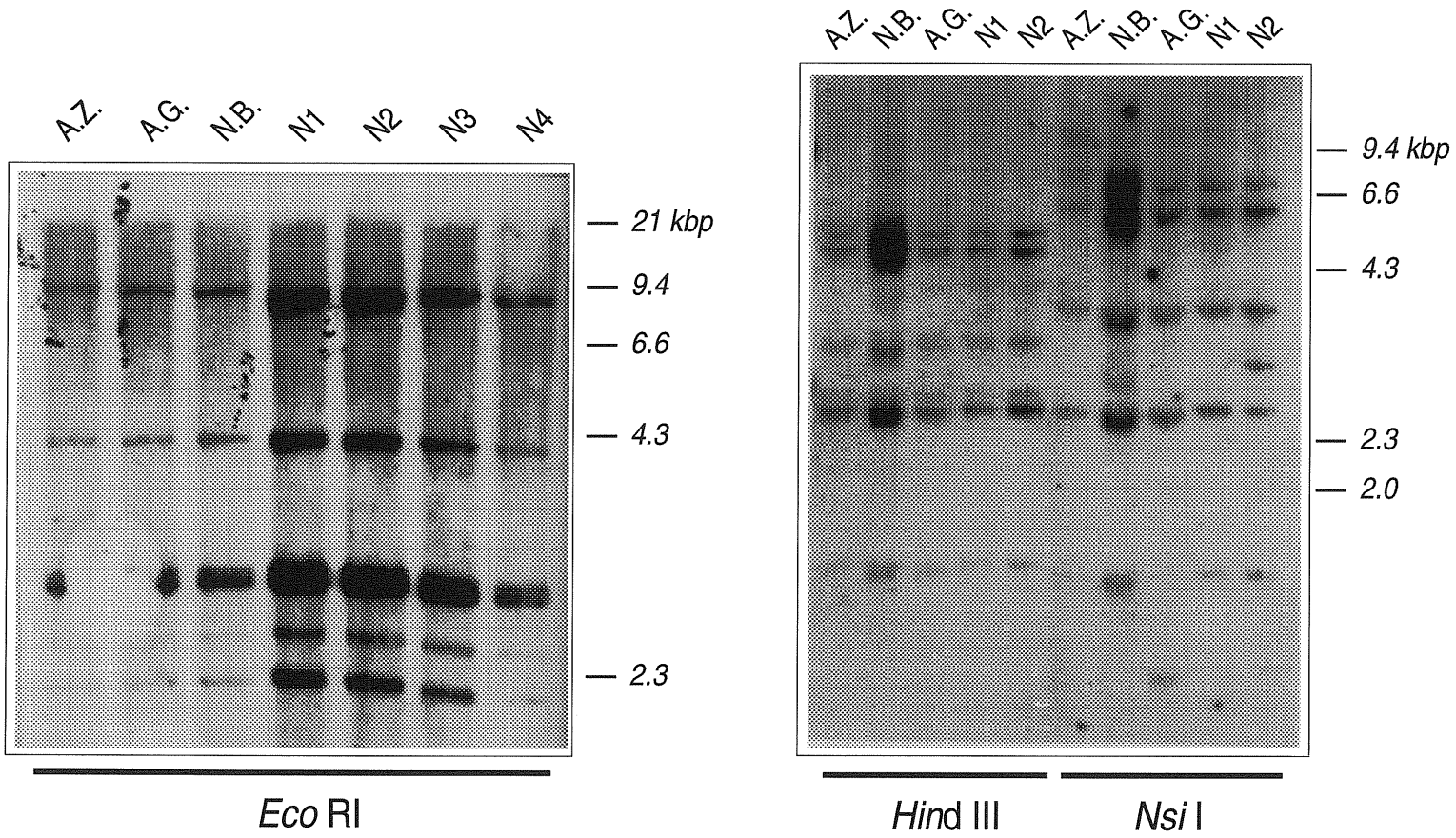


Figure 10. Southern blotting

Total genomic DNA obtained from B-lymphoblasts of the three X-CGD patients (A.Z., A.G. and N.B.) and normal unrelated controls (N1-N4) were digested with *Eco RI*, *Hind III* and *Nsi I*, as indicated, and probed with a DNA fragment encompassing the coding sequence of the gp91-*phox* cDNA.
The *Nsi I* restriction enzyme detects two sets of polymorphic DNA fragments (of 2,9 or 2,5 kbp and 1,7 or 1,3 kbp respectively; compare N1 and N2).

2.2 Sequence analysis

To find out the precise lesions responsible of the CGD phenotype in the patients, the entire protein coding sequence of gp91-*phox* gene was determined. The cDNAs obtained by reverse transcription from the RNA of the three patients were cloned in a pCR™ II vector. To facilitate cloning we divided the whole cDNA in five segments as detailed in material and methods. Sequence analysis showed (table 7) the presence of point mutations in the three patients. In patient A.G. there is an insertion of a "T" at nucleotide position 404 inside the exon 5 coding sequence, while the sequence derived from patient A.Z. reveals an "A" deletion at nucleotide 1330, within the splice acceptor site of exon 11. In patient N.B. a single "G" deletion was found at coding nucleotide 40. In patient A.G. the "T" insertion predicts an early stop codon at position 133 and a frameshift. In patient N.B. the "G" deletion causes a stop codon at position 21 and a frameshift. The third patients, A.Z., carriyng the "A" deletion, has a stop codon at position 501 and a frameshift.

An accurate analysis was done to exclude that the observed mutations were not the product of Taq polymerase misincorporation during the PCR amplification process. Since the reported mutations in A.G. and A.Z. cause the disappearance of the recognition sites for the restriction enzymes *Ava I* and *Bgl II* respectively, we analyzed newly amplified PCR fragments for the presence of these mutations. A new preparation of total RNA from patients and healthy controls was obtained and this RNA was than retrotranscribed, amplified and digested with the two previously mentioned enzymes. This experiment confirmed the previously obtained data.

In patients N.B., the presence of the mutation was confirmed by direct sequence analysis of a different clone, derived from another

	Patient: N.B.	*Patient:* A.G.	*Patient:* A.Z.
normal sequence	14 | Phe Val Ile *ex. 1* TTT GTC ATT	130 | Ala Arg Val Asn *ex. 5* GCC CGA GTC AAT	439 | Lys Ile Tyr *ex. 10* AAG ATC TAC *ex. 11*
mutated sequence	TTT ***TCA*** ***TTC*** Phe ***Ser*** ***Phe*** — ***STOP*** | 21	GCC ***CTG*** ***AGT*** ***CAA*** Ala ***Leu*** ***Ser*** ***Gln*** — ***STOP*** | 133	AAG ***TCT*** ***ACT*** Lys ***Ser*** ***Thr*** — ***STOP*** | 501

Table 7. Mutations of gp91-phox

The detected mutations in the cDNA of the three patients analyzed in this study are shown and compared to the normal sequence (upper part). Point deletions in N.B. and A.Z. and one base addition in A.G. lead to frameshift and insertion of premature STOP codons, as indicated. In patient A.Z., the mutation occurs at the first nucleotide of the acceptor splice site at intron - exon 11 boundary (the extremities of the two exons are boxed). Out-of-frame sequences are typed in bold letters. Numbering is referred to aminoacid sequence.

retrotranscription/amplification reaction on an independent RNA sample.

3. Northern Blotting

Northern blot analysis of the transcriptional pattern of gp91-*phox* gene was performed on total RNA extracted from the lymphoblastoid cell lines of the three patients and of three normal controls. Twenty μg of total RNA were resolved on a denaturing agarose gel, blotted on nylon membrane and then probed with two different probes for the gp91-*phox* and the β-actin transcripts. The gp91-*phox* probe is a *PstI-SacI* fragment of gp91-*phox* cDNA derived from the pBsII-Ks plasmid. The other probe (β-actin) is 226 bp PCR amplification product obtained by primers BA1/BA4 (see material and methods for details). The β-actin probe was used as a control for the integrity and amount of the extracted RNA.

From the northern blotting shown in figure 11 it is possible to see that in patients N.B. and A.G. bands similar in amount and mobility to those of normal controls are present. It is also possible to observe the presence of a barely detectable band migrating faster than the normally predominant transcript. This minor, smaller transcript is likely to be the product of an alternative start site normally found in phagocytic cells (197). From these data it is possible to conclude that the gp91-*phox* mRNA in these two patients is normal in size and quantity. As far as patient A.Z. is concerned, a markedly reduced amount of gp91-*phox* mRNA is evident. However, the transcript length seems to be normal. It is also present, although very reduced in intensity, the smaller gp91-*phox* transcript usually found in healthy controls.

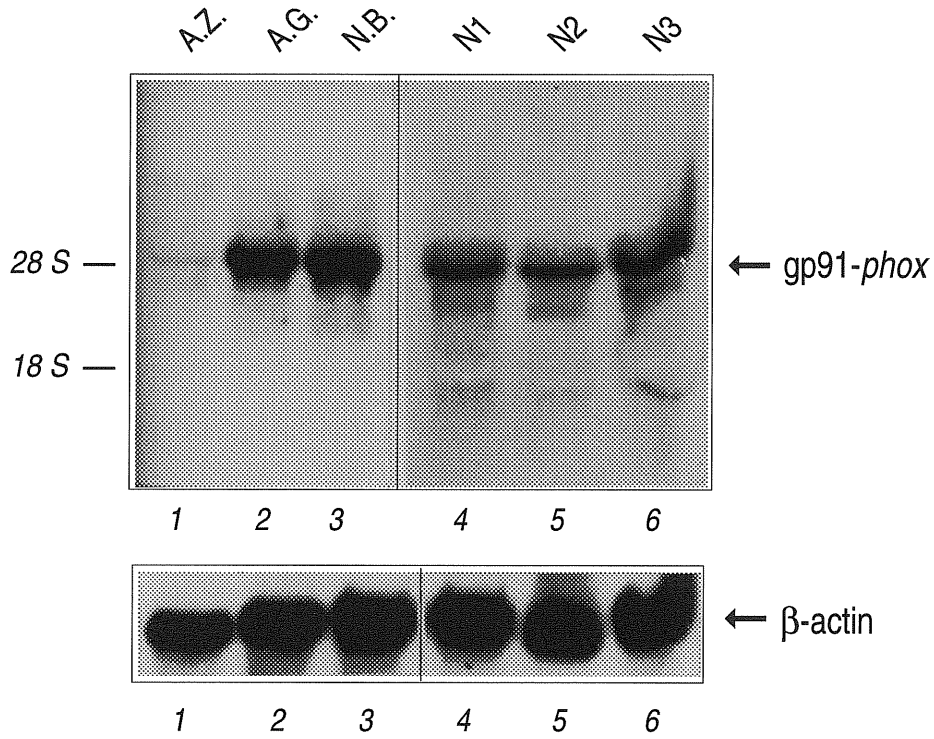


Figure 11. Northern blotting of RNA from lymphoblastoid cell lines derived from CGD patients

Blots of total RNA from patients and normal unrelated controls (N1-N3) were hybridized to a labeled cDNA fragment encompassing the entire coding region of the gp91-*phox* gene and subsequently to a labeled cDNA fragment for β-actin for normalization. The markedly reduction of gp91-*phox* mRNA in patient A.Z. is evident.

4. Quantitative PCR

The next approach in the characterization of the molecular defects responsible for the disease in the three patients was the precise quantification of the gp91-*phox* transcript. The need for setting up a precise and sensible method for the quantification of the gp91-*phox* gene is mainly due to two different reasons:

a) the northern blotting experiments could be prepared only on RNA extracted from lymphoblastoid cell lines, since only limiting amount of blood could be drawn from the patients. Therefore, confirmation of transcript abundance in granulocytes was still required.

b) in the perspective of a gene therapy program aiming at the correction of the genetic defect by introducing the gp91-*phox* cDNA in hematopoietic stem cells, we were interested in setting up a sensible system able to detect the expression of the newly transduced gene.

4.1 Principles of competitive polymerase chain reaction

Due to its extraordinary sensitivity, PCR is the method of choice for the detection of nucleic acid present in low abundance in biological samples. Quantitation by PCR, however, is problematic, since the final yield of the amplification reactions can be affected by several parameters, some of which can be hardly controlled by the operator, even in the most controlled experimental conditions. Since the final product derives from exponential amplification of the starting template, minor differences in amplification efficiencies (especially in the first cycles) will result in large differences in the overall product yield, especially if the amount of initial

template is low. For this reason, a reliable method for quantification is essential. Several authors have observed a linear relationship between input template and amplification product within the exponential range of amplification (202), (228), (262), (70), (43), (11), (253). This range, however, is strictly dependent on the abundance of the starting material (the more abundant the material, the shorter being the range (44), and is heavily influenced by differences in samples preparation, machine performance, reaction conditions, and presence of inhibitors. Similar problems must be faced by methods using limiting dilution analysis of the sample (261). For all these reasons, although semiquantitative data can be obtained readily with dilution curves, quantitative analysis is cumbersome.

An approach to overcome these tube-to-tube variations has been the co-amplification within the same tube of a reference template, being a single copy cellular gene (133), (208), (91), (196) or an ubiquitously expressed mRNA (43). The principles of the technique is that any variable influencing amplification should affect both the reference and the template similarly, if the reaction is maintained into its exponential phase. However, even if all the amplification parameters for each primer set are previously empirically determined, nevertheless the nature of the amplified sequence and of the primers have a largely unpredictable influence on the efficiency of amplification.

For all these reasons, the most reliable approaches to quantitative PCR are those based on co-amplification of reference templates that share with the target sequence the same primer sites and the near totality of the amplified sequence, so that the two templates compete for the same primer set and subsequently amplify at the same rate (competitive PCR). The two amplified products can be recognized because of their different lengths (257), (204), (95), (300), or for the presence of a mutation in the

competitor which creates a novel restriction site (24), (95) or can be resolved by temperature gradient gel electrophoresis (109).

However, since natural competitor sequences are not often available, the major problem suffered by competitive PCR is the construction of competitors, which can be often a tedious and long work of mutagenesis and cloning. The relevant characteristics of the methodology developed is the use of a competitor RNA molecule which shares the same sequence as the target mRNA molecule, except for a 20 nt insertion in its middle, and directly derived from the amplification product by an application of the recombinant PCR technology (115), (116) without need for cloning.

4.2 Development of a competitive RT-PCR method for quantitation of low abundance mRNAs

This chapter describes the general set up of the method, which has extensive applicability to several other areas where precise quantitation of RNA is needed (such as quantitation of viral RNAs).

We have applied the principles of competitive RT-PCR method to the quantitation of RNA. The technique consists in the co-amplification of the RNA sample to be quantified with different known amounts of a competitor RNA molecule that contains the same sequence (including primer recognition sites) of the target molecule except for a small insertion of 20 nt in the middle. The two RNA species compete for amplification and, at the end of the reaction, the amplification products for the two species can be simply resolved by gel electrophoresis. There are several advantages in the utilization of this competitive RT-PCR procedure in comparison to other methods of RNA quantitation by RT-PCR: 1) quantification performed by using an mRNA competitor overcomes the

problems of unpredictable RT inhibition dependent on the quality of extracted RNA; 2) the initial ratio between the number of molecules of the target and the competitor is maintained constant throughout both the RT and PCR steps according to the principles of competitive PCR (300), (95), (79); as a consequence, quantitation is not affected by the overall yield of either reaction; 3) the ratio between the two products is maintained even in the plateau phase of amplification (260), avoiding the need for hybridization or labeling procedures, even when starting from very limited amounts of templates; 4) the ratio between the amounts of the two species is independent from the formation of aspecific amplification products ; 5) competitor fragments can be easily obtained by direct transcription of DNA amplification products, without the need for cloning of the template DNA; 6) in contrast to other competition methods (247), (101) the amplification products can be simply resolved by gel electrophoresis and detected by ethidium bromide staining.

The competitor RNA was obtained according to the procedure outlined in figure 12. One oligonucleotide complementary to the RNA sequence in close proximity to an intron/exon boundary (in order to avoid subsequent amplification from genomic DNA contaminating the RNA sample) was chosen and utilized for cDNA synthesis with reverse transcriptase (step a). Next, the same oligonucleotide and a novel oligonucleotide with its 3′ portion identical to RNA sequence spaced few hundred nucleotides upstream of the first one, and a 5′ unrelated tail containing the recognition sequence for T7 RNA polymerase were used for a first PCR amplification step, resulting in a DNA fragment identical to a portion of the RNA plus T7 signal at one extremity (step b). At this point, two novel oligonucleotides (internal primes) were utilized, one for the sense and one for the antisense strands, corresponding to contiguous sequences at the 3′ ends and bearing a 20 nt long unrelated sequence at the

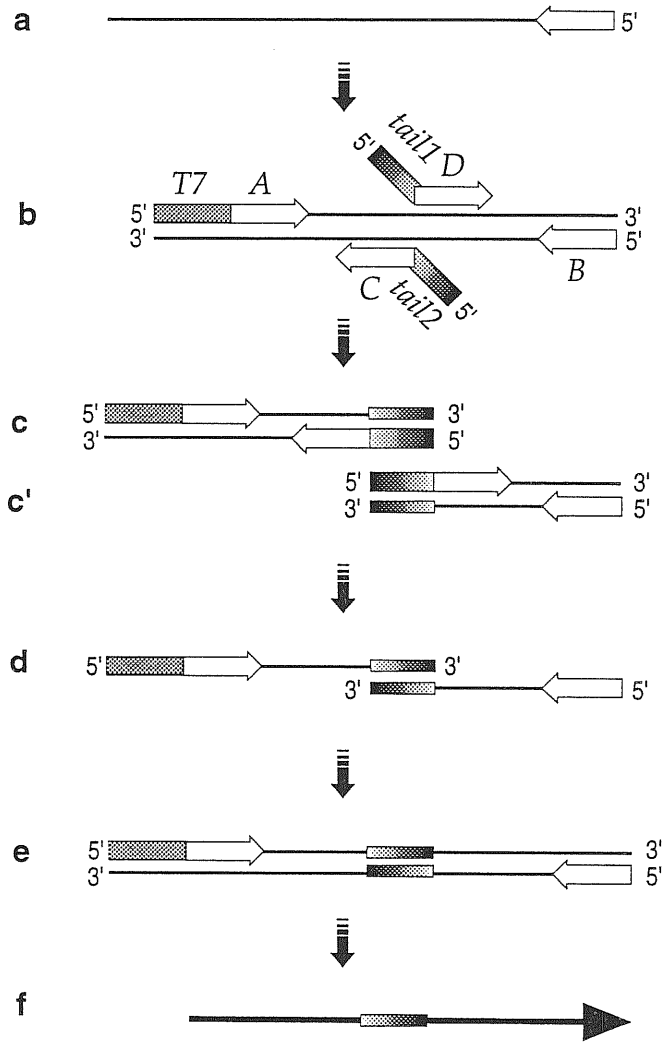


Figure 12.

5′ end. The two unrelated sequences were chosen in order to be complementary to each other. These primers were used in two separate PCR amplification reactions (step c-c'), in each of which one of the external primers was utilized as opposite strand primer. The two PCR products, which contain two contiguous sequences of the original fragment plus the 20 bp unrelated sequence at one extremity, were eluted, mixed together, annealed and amplified with the external primers (step d-e), to obtain a DNA fragment corresponding to the original amplification products plus the 20 bp in the middle. RNA can be directly obtained from this fragment by run-off in vitro transcription with T7 RNA polymerase (step f). The amount of RNA obtained was than loaded on a denaturing polyacrylamide gel and eluted. After elution the competitor was digested with RNase free-DNase I in order to completely eliminate any possible contamination by the cDNA template. The RNA obtained by this method can be directly used in competitive RT-PCR experiments, since it is identical to the target sequence to be amplified, except for the 20 nt insertion in its middle. The procedure utilized for the construction of the competitor RNAs is detailed in material methods.

4.3 RNA competitor or DNA competitor

The quantification of an RNA sample can be performed using an RNA competitor or a DNA competitor. In the first case the RNA competitor is added to the sample just before the reverse transcription step, while in the second a DNA competitor is added after reverse transcription step and before PCR amplification. Actually it is easier to work with a DNA competitor as it does not undergo degradation as fast as RNA does: therefore it is possible to prepare large stocks of competitor to be used for all the quantification experiments. On the contrary, RNA

competitor has to be prepared several times during quantification experiments and this is often a time-consuming work. For this reason, before starting the quantification experiment, we decided to verify the need for using an RNA competitor instead of a DNA competitor.

To investigate the variability of the RNA extraction step, we performed the following experiment. β-actin transcript was quantified in five unrelated samples using either DNA or RNA competitor. RNA samples were all prepared with the same method (46) but in different periods. RNA from samples 1, 2 and 3 was extracted in the same day, while RNA from samples 4 and 5 was extracted one week and two weeks later, respectively. DNA competitor was obtained by reverse transcription of a known amount of RNA competitor (10^8 molecules/μl). The RT product was than progressively diluted (up to 10^5 molecules/μl). These dilutions were used as DNA competitor in the quantification experiment. The RNA competitor was obtained as already described.

Results of the double quantification are shown in table 8. For three samples (n.1, n.2 and n.3) the ratio between the quantification with the RNA competitor and with the DNA competitor is roughly two-fold. For the other two samples the ratio is eight (n.5) and four-fold (n.4). From these data we can deduce that in samples n.1, n.2 and n.3, 50% of the RNA molecules have been retrotranscribed after the RT step while in samples n.4 and n.5, 25% and 12,5% respectively have been retrotranscribed. Since the same reagents and the same experimental conditions were used for reverse transcription of all samples, we can conclude that the different quantifications obtained by the two methods depend on the RNA quality. Interestingly, samples 1, 2 and 3 prepared in the same day, show the same differences between the two quantification methods. This observation further supports the idea that the quality of the RNA to be tested depends

Samples	RNA competitor number of molecules/µl	DNA competitor number of molecules/µl
N.1	$6x10^6/\mu l$	$3x10^6/\mu l$
N.2	$2.4x10^7/\mu l$	$1.8x10^7/\mu l$
N.3	$3x10^6/\mu l$	$1.5x10^6/\mu l$
N.4	$1.9x10^7/\mu l$	$4.8x10^6/\mu l$
N.5	$4x10^6/\mu l$	$5x10^5/\mu l$

Table 8.

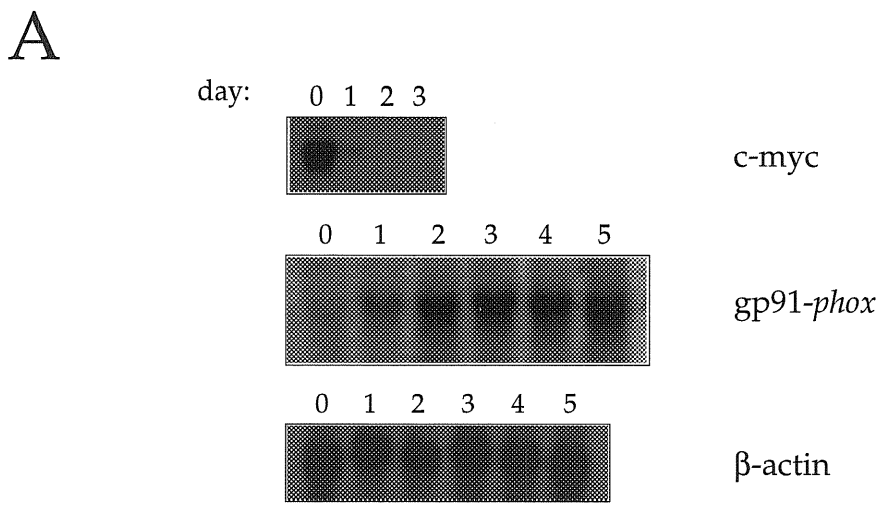
on the extraction procedure and greatly influences the reverse transcription efficiency.

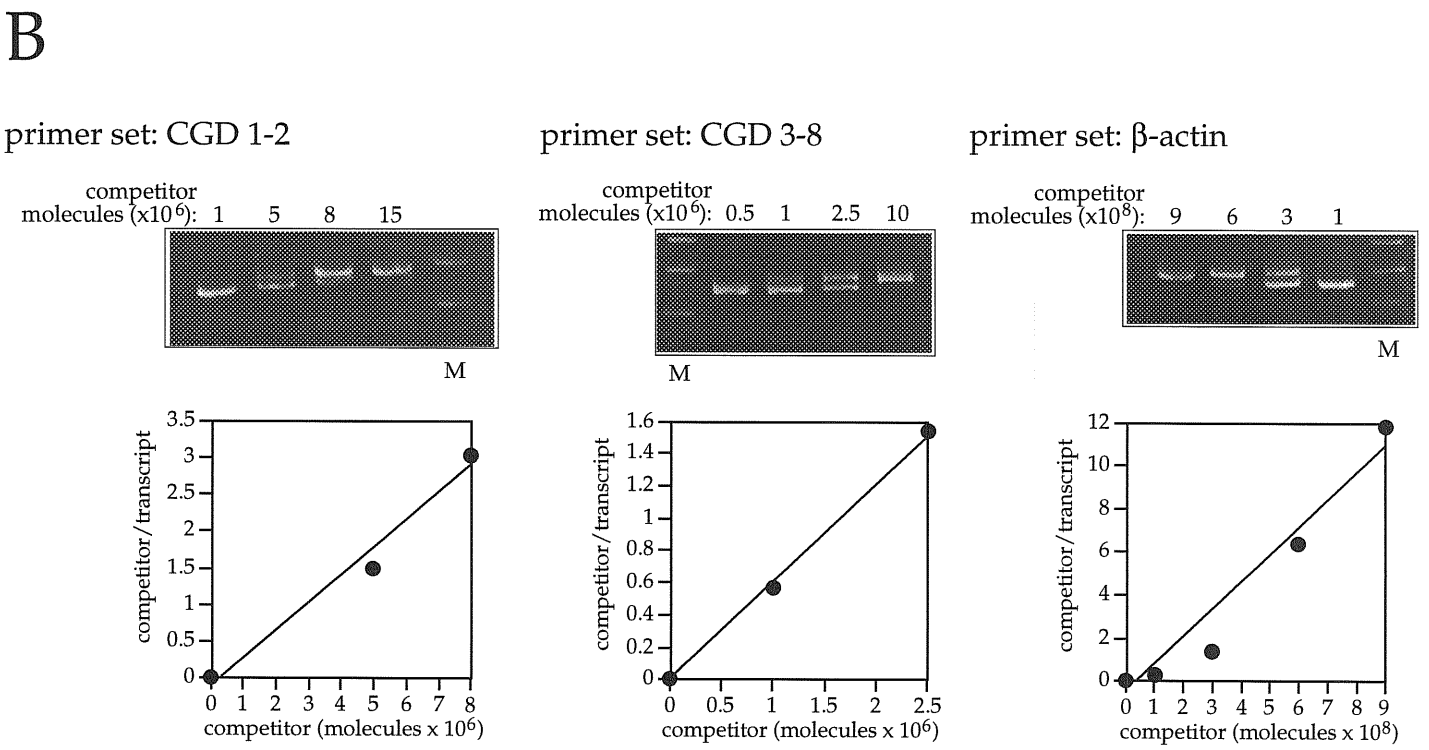
This experiment points out the importance of using an RNA competitor instead of a DNA competitor. The efficiency of reverse transcriptase can greatly vary from sample to sample depending on the quality of RNA. To overcome this variation it is necessary to add the RNA competitor before RT: in this case, both RNA species (target and competitor) will be retrotranscribed with the same efficiency. The ratio between the two molecular species will remain constant throughout the overall RT-PCR procedure.

4.4 Quantitation of gp91-phox mRNA in differentiating HL-60 cells.

Before quantifying the gp91-*phox* transcript in normal and affected individuals, we decided to perform the same experiment on progressively differentiating HL60 cells. The aim of this preliminary experiment was to evaluate the sensibility and reliability of the detection system we developed. HL60 cells undergoes a low rate (1 to 5% of cells) of spontaneous myeloid differentiation in vitro (56). Incubation with polar solvents, such as retinoic acid, induces markedly increases differentiation to morphologic polymorphonuclear leukocytes. We studied the expression pattern of *c-myc* and gp91-*phox* genes during differentiation by northern blotting assay. In addition, we quantified the gp91-*phox* transcript by RT-PCR in order to compare these data with those obtained by northern blotting.

Three competitor RNAs were constructed for competitive RT-PCR experiments on progressively differentiating HL60, as described in the previous section and detailed in materials and methods. One of these competitors allows absolute quantitation of the β-actin mRNA (an





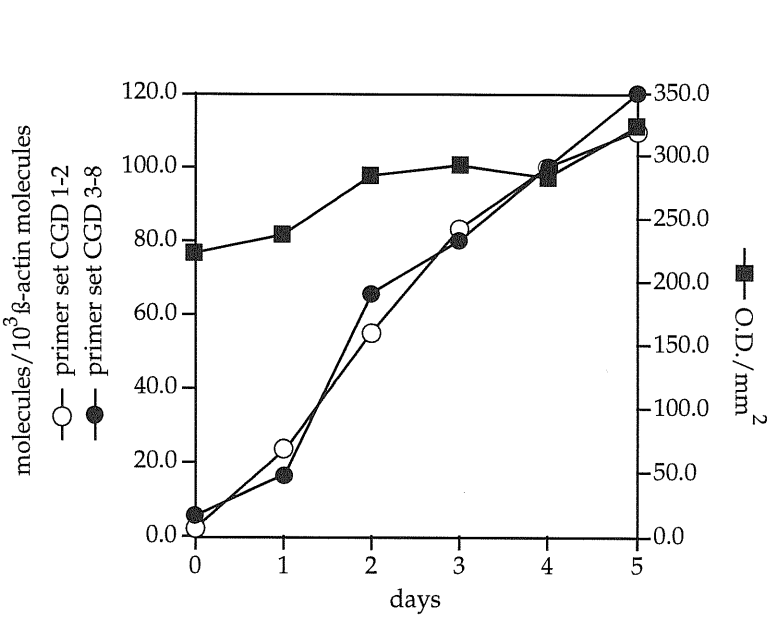


Figure 13 (legend on following page)

Figure 13.

Quantitation of gp91-*phox* in differentiating HL60. **A.** Northern blotting. Twenty μg of total cellular RNA of HL60 cells extracted at days 0-5 after induction of differentiation was hybridized to a gp91-*phox* cDNA probe (a *PstI-Sac*I, fragment of gp91-*phox* cDNA), to a β-actin probe (the RT-PCR product obtained with primers BA1 and BA4), and to probe for the c-*myc* gene (a 1500 bp *EcoRI* DNA fragment, including the first and second exons). **B.** Competitive RT-PCR. A fixed amount of the RNA samples from HL60 cells at day one after induction of differentiation was mixed with increasing amounts of competitors (as indicated on top of gels) and submitted to RT-PCR; the amplification products were resolved by polyacrylamide gel electrophoresis and stained with ethidium bromide (indicated by arrows). For each amplification, the ratio between the intensities of the bands (evaluated by densitometric scanning) was plotted against the amount of competitor added (lower part). As expected, the points are fitted by a straight line emanating from the origin. M indicates the molecular weight marker lane. **C.** Results of quantitation. The amount of molecules of gp91-*phox* mRNA detected by two independent quantifications (primer sets and competitors CGD 1-2 and CGD 3-8) in RNA samples from HL60 cells at different days of differentiation are expressed per 10^3 molecules of β-actin mRNA (scale on the left side). For comparison, the results corresponding to the ratio between the intensity of the bands of gp91-*phox* and β-actin of the blot of panel A are shown (scale on the right side).

abundant messenger present in almost any cell as a constituent of the cell cytoskeleton), the other two allow the quantitation of the gp91-*phox* mRNA. The two competitors constructed for the quantification of gp91-*phox* are named CGD1-2 and CGD3-8. Competitor CGD 1-2 allows quantitation of a region close to the 5' end of the gp91-*phox* transcript while competitor CGD 3-8 allows the quantification of a region corresponding to the 3' end of the transcript (see material and methods for details). The use of two different competitors for gp91-*phox* allowed us to validate the quantification procedure, providing two independent quantitations of the same transcript.

We treated undifferentiated HL60 with retinoic acid and dimethyl formamide for five days: in these conditions, cells stop dividing, switch off transcription of the c-*myc* gene and start expressing detectable levels of the gp91-*phox* mRNA (figure 13 panel A). The amount of the gp91-*phox* mRNA in the same RNA samples utilized for the Northern blots was determined by competitive RT-PCR; an example of the procedure utilized is shown in figure 13 panel B for the RNA at day 1. A fixed amount of each mRNA sample was mixed with increasing amounts of competitor RNAs and submitted to the RT-PCR procedure. After amplification, the reaction mixtures were separated by polyacrylamide gel electrophoresis, and the intensity of the bands determined by densitometric scanning. Figure 13 panel B reports the results of the quantitation obtained, by plotting the number of competitor molecules added against the ratio of the two amplification products obtained. As expected from the theory of competitive PCR (79), the points are fitted by a straight line; as a consequence, the amount of competitor corresponding to a 1:1 ratio between the two products corresponds to the amount of input RNA in the sample. It should be considered that a single point of competition is theoretically sufficient to determine the exact target RNA concentration; as

a matter of fact, at least two or more points possibly including the range of transition from the excess of competitor to the excess of target (as shown in the figure 13) are required to obtain a precise quantitation.

The results of the quantitation of the gp91-*phox* mRNA molecules in the samples from the different days after initiation of the differentiation treatment are shown in figure 13 panel C as expressed per 10^3 molecules of β-actin mRNA. From these data, it can be concluded that, in the absence of treatment, the gp91-*phox* transcript in HL-60 is more that 100 times less represented than the β-actin transcript, and that, upon stimulation, it increases linearly up to day 5 of more that 50 fold. As it is evident from the figure 13, the independent quantitation of the same transcript with two different competitors gives equivalent results. On the contrary, densitometric scanning the Northern blot for figure 13 panel A shows saturation of the intensity of the bands after day 2, a known intrinsic limit of this method of quantitation (exposure of the same blots for less time, on the contrary, results in under-representation of the bands of the first two days; not shown). Furthermore, 200 times less RNA (100 ng versus 20 μg) was utilized for each competition as compared to the amount used for the blots. Altogether, these results indicate that competitive RT-PCR is a very accurate, sensitive, and reproducible method for quantitation of low abundance mRNAs.

4.5 Quantitation of the gp91 mRNA from samples of CGD patients and normal individuals

By the above described competitive RT-PCR procedure, the gp91-*phox* transcription pattern was studied both in peripheral granulocytes and in lymphoblastoid cell lines from healthy donors and from affected patients. Ten milliliters of blood were drawn from three normal

			Primer set			*Ratio*	
			β-actin (molecules x10 6/ l)	CGD 1-2 (molecules x10 3/ l)	CGD 3-8 (molecules x10 3/ l)	CGD 1-2/ β-actin (x10 3)	CGD 3-8/ β-actin (x10 3)
CGD	B-LCL	N.B.	3,000	10,000	6,000	3.33	2.00
		A.Z.	100	0.03	0.1	0.00	0.001
		A.G.	200	400	500	2.00	2.50
	Granulocytes	N.B.	80	90	90	1.12	1.12
		A.Z.	600	30	30	0.05	0.05
		A.G.	70	150	100	2.14	1.43
normal	B-LCL	N1	500	1,500	1,000	3.00	2.00
		N2	800	1,000	800	1.25	1.00
	Granulocytes	n1	100	800	600	8.00	6.00
		n2	50	400	300	8.00	6.00
		n3	100	900	700	9.00	7.00

Table 9. Results of quantification of mRNAs for β-actin (primer set β-actin) and gp91-*phox* (primer sets CGD 1-2 and CGD 3-8, providing independent quantitations) in samples from peripheral blood granulocytes and B-lymphoblastoid cell lines (B-LCL) of normal individuals (N1-N2 and n1-n3) and X-CGD patients (N.B., A.Z. and A.G.).

The two righmost columns reports the amount of gp91-*phox* mRNA after normalization for β-actin levels.

individuals and three affected children (A.G., A.Z., N.B.). Peripheral blood granulocytes were recovered after red blood cells ipotonic lysis and dextran sedimentation. Aliquots of ~$5x10^6$ granulocytes were used for RNA extraction.

Different amounts of competitor RNAs for β-actin and gp91-*phox* (from 10^1 to 10^7 molecules for gp91-*phox* and from 10^6 to 10^9 molecules for β-actin) were added to each sample in independent quantitation experiments. These competitor species, and the corresponding cellular mRNAs, were then submitted to reverse transcription and PCR amplification. As detailed above, by this method the addition of competitor RNAs before the PCR amplification cycle also allows the control of the efficiency of the retrotranscription step.

The two amplification products for competitor and template were then resolved on a polyacrylamide gel, stained with ethidium bromide and photographed. The equivalence point (i.e. the concentration of competitor which roughly gives rise to an amplification product of the same amount as the cellular mRNA product) was estimated by scanner analysis of the band intensities, and a more precise quantitation of the absolute amounts of cellular mRNAs was then obtained by a second competitive RT-PCR experiments with two-fold dilutions of competitor centered around the equivalence point.

The results of quantitation of the absolute amounts of gp91-*phox* and β-actin mRNAs in peripheral granulocytes and in lymphoblastoid cell lines from normal individuals and the three CGD patients are shown in table 9. Since β-actin is assumed to be expressed at constant levels in several cell types (including granulocytes), it can be used as a standard for quantitation. It is therefore possible to make a comparison between the data obtained from different subjects and from different cell types.

Considering the mean value of gp91-*phox* ($6.16x10^2$ molecules/μl) and β-actin ($8.333x10^4$ molecules/μl) quantification in normal granulocytes among the different samples, it is evident that the gp91-*phox* gene is expressed more than 100 times less than the β-actin gene. A lower level of mRNA expression (roughly 4 time less) was found in lymphoblastoid cell lines as compared to granulocytes. These data are not surprising if it is considered that only granulocytes are physiologically committed to the production of superoxide, while B cells can generate superoxide only after the in vitro immortalization. These data are in agreement with previous published works (294), (217).

If we compare the data obtained from the normal lymphoblastoid cell lines and those from the three patients, it is evident that, while two patients (N.B. and A.G.) show normal level of gp91-*phox* expression, one (A.Z.) shows a greatly impairment in expression (roughly 10^4 times less than in normal controls). It is worth mentioning that these data are obtained after normalization to β-actin, thus excluding the possibility that a partial degradation of A.Z. RNA samples had occurred.

As far as the data in granulocytes are concerned, it results that expression is again highly impaired in patients A.Z.. On the contrary, the other two patients show only a slight reduction of the transcript.

These data further confirm that B-cell lines reflect the physiological and pathological events of NADPH oxidase regulation.

5. Western blotting analysis.

After having studied the genomic DNA and the transcriptional pattern of the gp91-*phox* gene, we decided to investigate the presence of cytochrome b_{558} on lymphoblastoid cell line membranes of the patients. For this purpose we used the two monoclonal antibodies MoAb 48 and

MoAb 449 (291) raise against the gp91-*phox* protein and p22-*phox* protein respectively.

Lymphoblastoid cell line membranes, from the three patients and from normal controls, were prepared from 10^8 cells. After cells sonication, membranes were separated on sucrose gradients and divided in four fractions. Each fraction was then frozen at -80°C.

The presence of gp91-*phox* protein lymphoblastoid cell line membranes of a healthy donor were first investigated to set up blotting conditions. An aliquot of cellular membranes was also digested by endoglycosidase F which is able to cleave the N-linked oligosaccharides from the gp91-*phox* protein (142). By this experiment, it was possible to detect both the glycosylated form of gp91-*phox* protein and the core protein (figure 14). The glycosylated form of the protein typically appears as a smear at 70 to 100 kDa due to the variable grade of glycosylation of the protein core. On the contrary, the endoglycosidase F digested protein, lacking the oligosaccharide component, appears as a clear band of 55 kDa. As expected, the amount of the gp91-*phox* subunit in B-cell lines was roughly 10 times less (55) than that found in granulocytes (data not shown). The same experiment was repeated by using membranes from lymphoblastoid cell lines of the patients. As shown in figure 14, no evidence for the gp91-*phox* core protein was seen in protein samples from CGD patients.

After checking for the presence of the gp91-*phox* protein, we probed the B cell lines membranes from healthy donors and from the patients for the presence of p22-*phox* protein, the other component of cytochrome b_{558}. MoAb 449 revealed (figure 15), although at different levels, the presence of the p22-*phox* in all the patients. This is rather unusual since in the majority of the examined cases of X91° CGD, both subunits are usually absent (211). However, other few cases are described in which p22-*phox* is

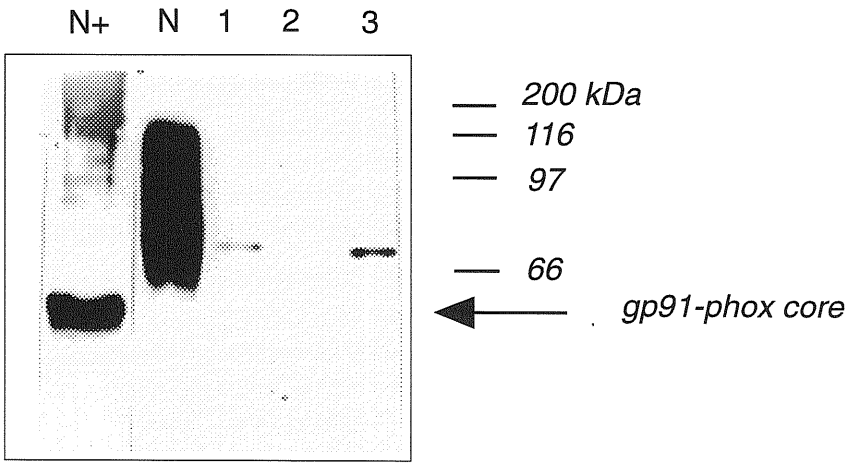


Figure 14.

Western blotting experiment: monoclonal Ig MoAb 48 detects both the deglycosylated (55 kDa)(N+; 50 µg) and glycosylated forms of gp91-*phox* protein in a normal control B cell line membrane preparation (N; 50 µg); no band is detectable in the membrane preparations of the three patients after digestion with endoglycosidase F (A.Z. , A.G. and B.N.; 50 µg each).

stable in the absence of gp91-*phox* (31), (291). To further investigate this point, we repeated the western blot experiment using another polyclonal antibody (Garcia, R., unpubblished data). This experiment confirmed the data obtained by the monoclonal antibody MoAb 449. Further investigations will be required to better clarify this point.

6. Luminol chemiluminescence test

To assess the oxidase enzymatic function of the cell lines of the three patients and of normal controls, a highly sensitive chemiluminescent assay was used. This test records the instantaneous level of H_2O_2 formed by the dismutation of O_2^- (316). The basis of the assay is the oxidation of luminol by peroxidase (which in the case of neutrophils may be myeloperoxidase) to form the activated aminoph-thalate anion. Owing to the fact that lymphoblastoid cell lines do not possess myeloperoxidase activity (220), horse-radish peroxidase was added to the components of the assay. To trigger superoxide generation in lymphoblastoid cell lines, phorbol-12-myristate-13-acetate (PMA) was used as an activator of protein kinase C. Chemiluminescence was monitored using a liquid scintillation instrument. Other systems (NBT reduction or DCFH oxidation) which are not specific for O_2^-/H_2O_2, are not suitable for lymphoblastoid cell lines (220).

For each lymphoblastoid cell line (from patients and healthy controls), 10^6 cells were tested before and after stimulation with PMA. Chemiluminescence readings were taken at 10-minute intervals from time 0 (no stimulation) to time 80'. The data obtained are reported in figure 16 where on the y axis, the chemiluminescence measure (counts per minute: cpm) is reported, while on the x axis the time is indicated.

From the data reported, it is clear that there is a great variability in

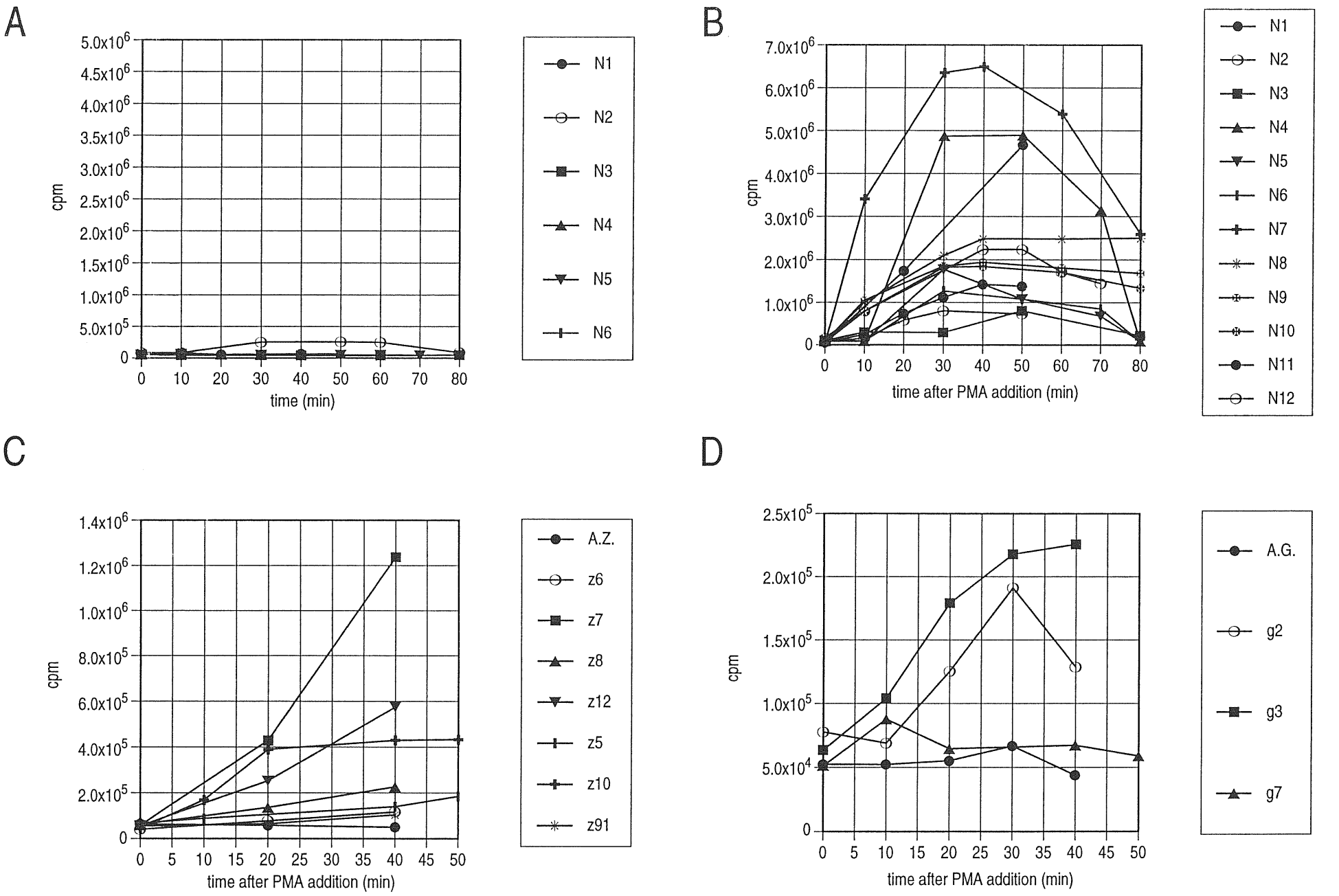


Figure 16 (legend on following page)

the O_2- production by normal control cell lines. For all normal controls the peak of chemiluminescence was reached 40 minutes after PMA stimulation. However, the intensities of the peaks vary among different samples. The chemiluminescence intensities ranges between 6.4 x 10^6 (n.7) cpm to 10^6 cpm (n.3). This variability may be due to the different biological behavior of each lymphoblastoid cell lines. From 40 minutes on, the chemiluminescence intensity decreases progressively for all the controls, although at different rates.

From the lymphoblastoid cell lines of all the patients, it was not possible to measure any increase in chemiluminescence intensity after PMA stimulation, thus demonstrating the inability of patients' cells to produce detectable levels of superoxide. The three patients, lacking the gp91-*phox* protein in western blotting analysis and having 0% of normal oxidase activity, can be classified as having the $X91^0$ CGD subtype, which is the most common subtype (50%) among all the cases so far described (235).

CORRECTION OF THE GENETIC DEFECT

The last part of this work concerns the correction of the genetic defect in the lymphoblastoid cell lines of two patients (A.Z. and N.B.). As detailed in the previous paragraph, B lymphocytes immortalized by EBV are of particular interest as they express the same NADPH oxidase complex as phagocytic cells and are able to generate superoxide in response to a number of stimuli including PMA (167), (103), (155). The magnitude of the response in these cells is lower of that of neutrophils but the overall enzymatic process appears to be the same. Since the lymphoblastoid cell lines from patients suffering of CGD show the biochemical and molecular defects similar to those of phagocytes, they can serve as an invaluable in vitro model of the disease.

We have used this model to prove the effectiveness of functional recostitution of oxidase activity by viral vector gene transfer. Although CGD is a uncommon hereditary disorder, it represents a very interesting model for a gene therapy approach, since both autosomal and X-linked forms are caused by a single gene defect and the cDNA encoding the different subunits has been cloned.

The data presented represent the first step in a gene therapy program aimed at the correction of the genetic defect in hematopoietic stem cells from affected patients. The fact that the NADPH enzymatic complex is expressed almost exclusively in myelomonocytic cell lineages and that bone marrow transplantation has been curative in some cases (60), indicates that the transfer of genetically corrected autologous gene in hematopoietic stem cells should correct the disorder.

1. Construction of a retroviral vector and transduction of X-CGD cell lines

The retroviral vector used in this study is the Moloney murine leukemia virus-based vector pBabeHygro (187). The vector is designed to trasmit the inserted gene with high efficency and to express it from the Mo-MuLV Long Terminal Repeat. The pBabe Hygro vector contains both retroviral LTR and the hygromycin resistance gene under the control of the SV40 promoter. It lacks most of the other viral *cis* elements except for a small portion of *gag* gene whose presence increases incapsidation efficiency (25), (10).

A 1.9 kb cDNA fragment, encompassing the entire coding region of gp91-*phox*, was cloned in the retroviral vector to obtain plasmid pBabeHygro/gp91-*phox*. Figure 17 schematically shows the genetic map of the retroviral vector carrying the gp91-*phox* cDNA coding portion.

With the purpose of obtaining a replication-defective virus for efficient transduction of target cells, we first introduced this plasmid into the murine ecotropic packaging cell line Ψ2 (170) using the calcium phosphate coprecipitation method (45). This helper cell line carries integrated into its genome the *trans* elements required for replication of the replication-defective viral vector. Furtheremore, the *env* gene integrated into its genome encodes for an envelope that will allow the retroviral vector to infect only rodent cells.

After viral vector introduction, cells were selected with 200U/ml of hygromycin B. These resultant colonies were pooled and their supernatant containing ecotropic recombinant retrovirus was used to infect the PA317 murine amphotropic cell line (181) in the presence of 8 mg/ml polybrene. This helper cell line contains an integrated *env* gene that allows infection of both rodent and non rodent cells, including human cells. The transduced PA317 cells were then replated and selected

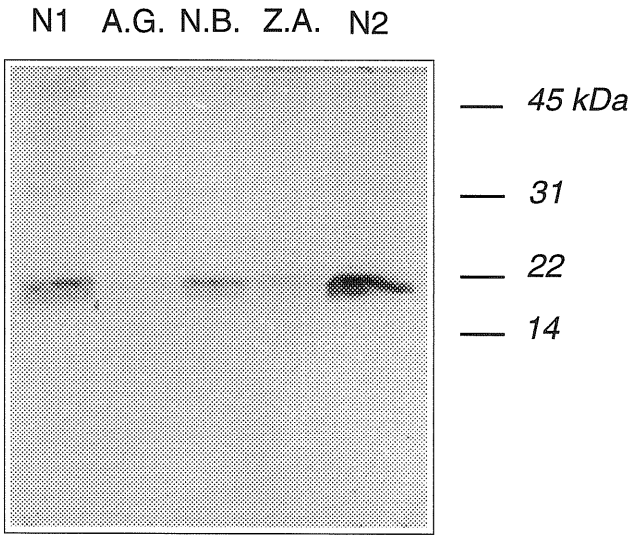


Figure 15.

Western blotting experiment: monoclonal Ig MoAb 449 detects a faint band corresponding to p22-*phox* protein in the membrane preparation of the B cell lines of the three patients (A.Z. 100 µg; A.G. 50 µg; N.B. 100 µg). N1 (50 µg) and N2 (60 µg) are normal controls.

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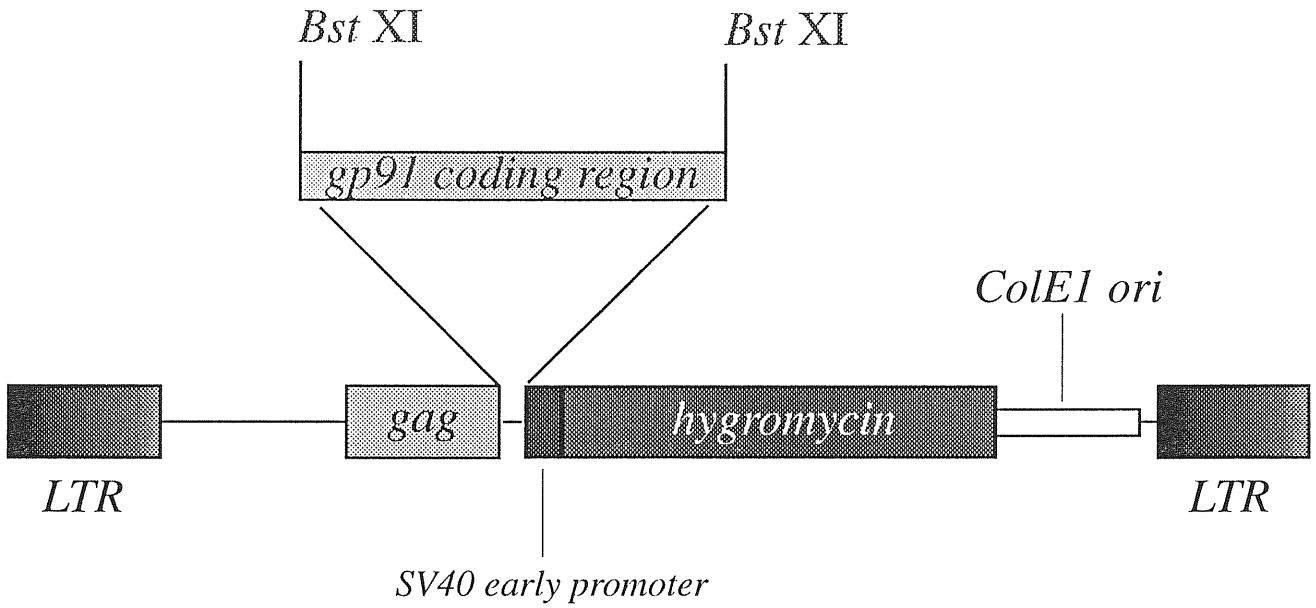


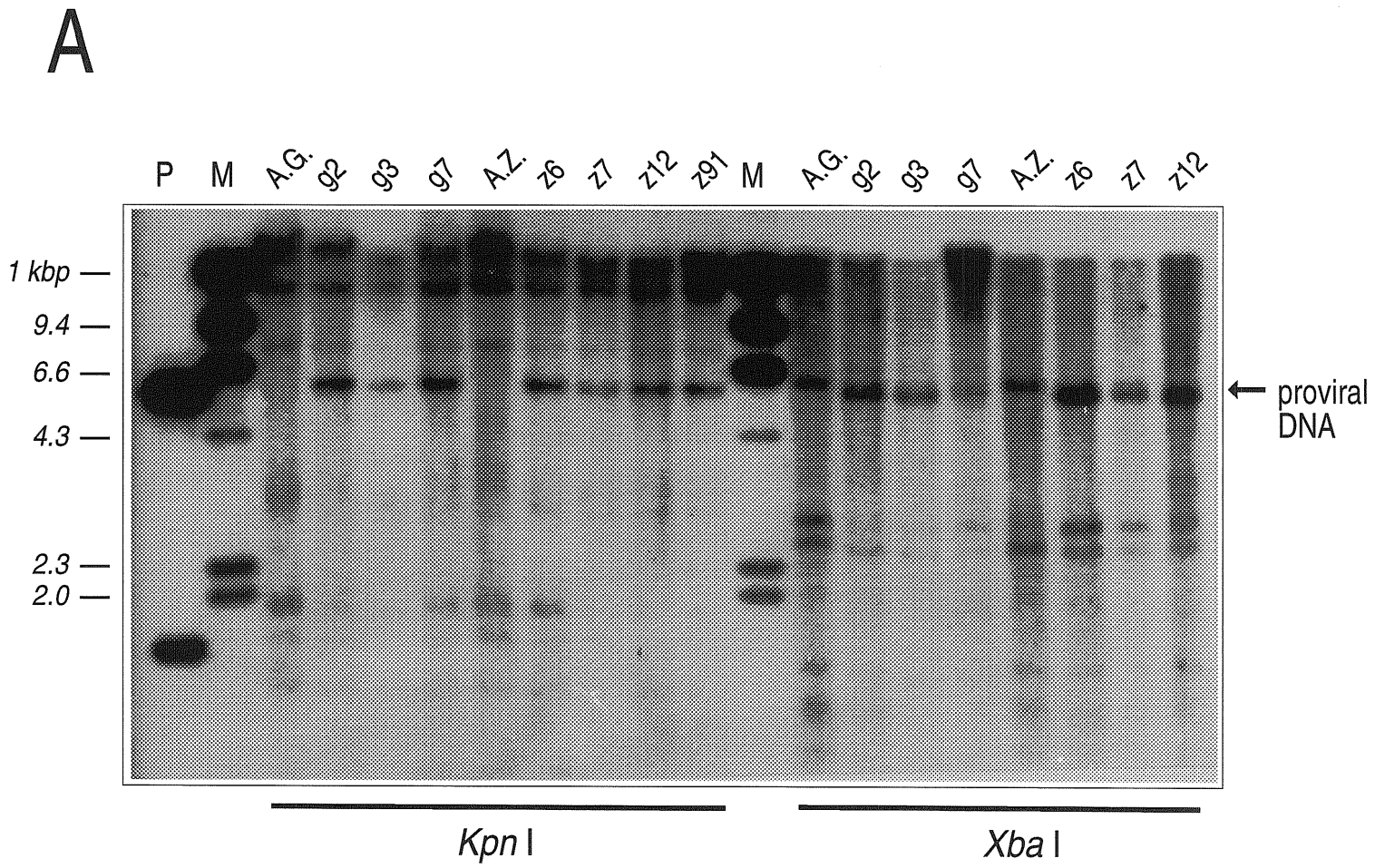
Figure 17.
Genetic map of the retroviral vector carrying the gp91-*phox* cDNA.

with 200 U/ml hygromycin B. Resistant colonies were isolated by ring cloning and expanded. The hygromycin resistant PA317 clones were than titered for the production of recombinant retrovirus by colony assay on murine fibroblast NIH3T3 cells. The clone producing the highest titer (1 x 10^4 CFU/ml) was used as a source of virus for transduction of B-lymphoblastoid cell lines of patients.

Nonhaderent A.Z. and A.G. lymphoblasts were transduced by cocultivation with the PA317 clone producing the best viral titer. Subconfluent PA317 cells were cocultured with 5 x 10^5 cells/ml from EBV B-lymphoblasts from the patients in the presence of 6μg/ml of polybrene. After overnight incubation, nonadherent cells were collected by centrifugation and resuspended in fresh RPMI 1640 medium supplemented with 20% FCS. Hygromycin B selection (200 U/ml) was started 48 hours later and continued for 4 weeks. Surviving cells formed typical cluster aggregates that were isolated and grown separately .

2. Genetic analysis of transduced B-cell lines

The transduced B-cells were then investigated for the presence and the expression of the gp91-*phox* vector. First of all, retroviral vector integration in the hygromycin-resistant clones was analyzed. To demonstrate vector integration, total genomic DNA was digested with *Kpn I* and *XbaI* both of which cut once in both proviral LTRs. The digested DNA was then hybridized to gp91-*phox* and hygromycin specific probes (see materials and methods for details). Figure 18 panel A shows the hybridization pattern obtained using the gp91-*phox* specific probe. A proviral band of the expected size with no apparent rearrangements is present in each clone. Analogous results were obtained using the hygromycin specific probe (data not shown). To estimate the number of



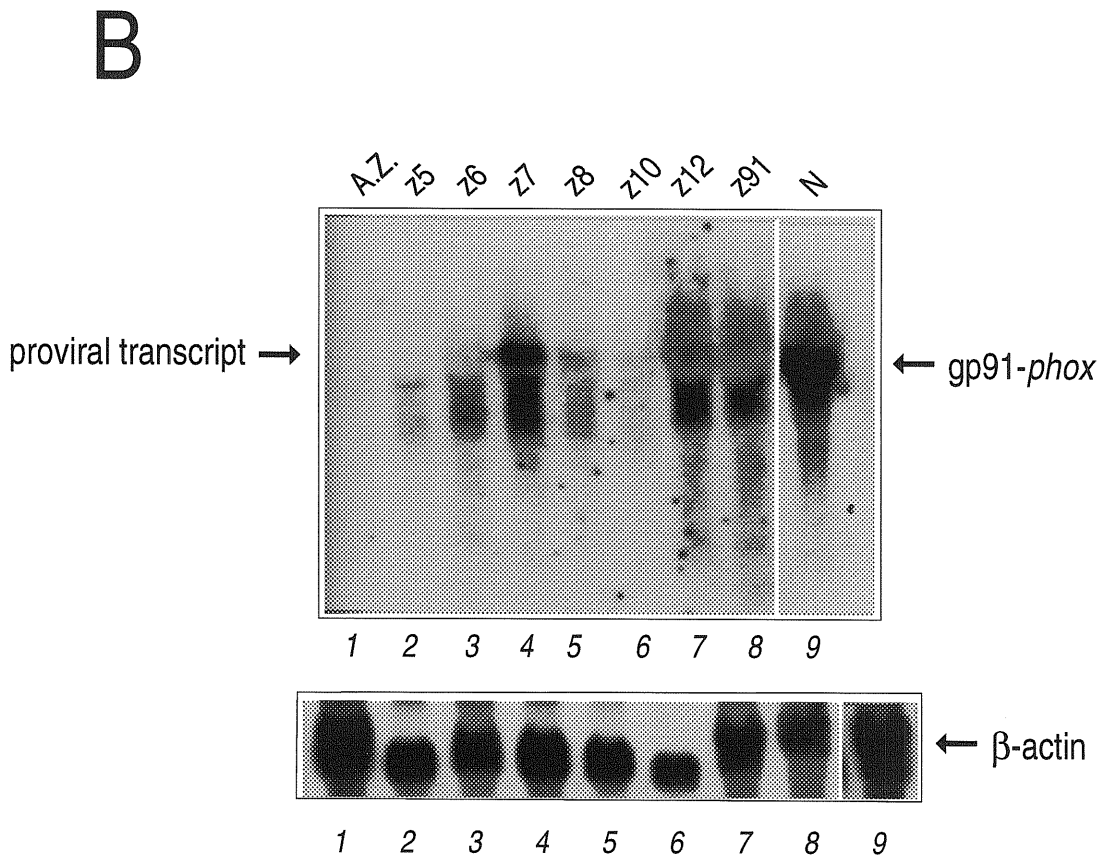


Figure 18 (legend on following page)

Figure 18. Analysis of transduced B-cell clones

Panel A. Southern blotting.
DNA extracted from transduced lymphoblastoid B cell clones from patient A.G. (clones g2, g3, g7)) and A.Z. (z6, z7, z12, z91) was digested with *Kpn* I or *Xba* I, both of which cut once in each viral LTR, and probed with a labeled gp91-*phox* cDNA fragment. The arrow indicates the proviral DNA band. A.G. and A.Z.: DNA from untransduced cell lines; M: labeled molecular weight marker; P: plasmid pBabeHygro/gp91-*phox* digested with *Kpn* I.

Panel B. Northern blotting.
RNA from transduced lymphoblastoid B cell clones of patient A.Z. was hybridized to a gp91-*phox* cDNA probe and subsequently to a β-actin probe. The arrow on the left side of the upper blot indicates the 7 kb specific proviral mRNA, the arrow on the right side indicates the endogenous 5.6 kb mRNA of a normal lymphoblastoid cell line (N). A.Z.: RNA from the untransduced parental B cell line.

vector copies integrated per cellular genome in the transduced B-cell lines, we compared the band intensity of the integrated vector with that of the endogenous gene considered as a standard. We can conclude that the vector is present in one or two copies per cellular genome.

Next step in the analysis of vector integration and function was the study of vector expression. For this reason, we performed a Northern blot assay using total RNA extracted from the transduced B-cell clones. The northern blot in figure 18, panel B, probed with gp91-*phox* cDNA, shows expression of the retroviral transcript in transduced B-cells from patient A.Z. Although the LTR-driven expression of gp91-*phox* transcript varies between clones, the levels of the specific transcript detected in the clones are comparable to those of normal control B-lymphoblastoid cell lines. Similar results were obtained for transduced B cell lines from patient A.G. (data not shown).

The last step in the study of the transduced clones was the detection of the newly synthetized gp91-*phox* protein. We investigated some of the transduced B-cell clones for the presence of the gp91-*phox* protein using the MoAb 48 (291). Membrane preparation was conducted as described above. The product of gp91-*phox* protein tipically appears as a smear at 70 to 100 kDa, due to the variable grade of glycosylation of the protein core. In order to obtain a stronger and homogeneous signal on immunoblot, the membrane fraction preparations were treated with endoglycosidase F that reveals the 55 kDa core protein. As shown in figure 19, the specific protein was evidentiated in two transduced clones that showed a high level of mRNA expression.

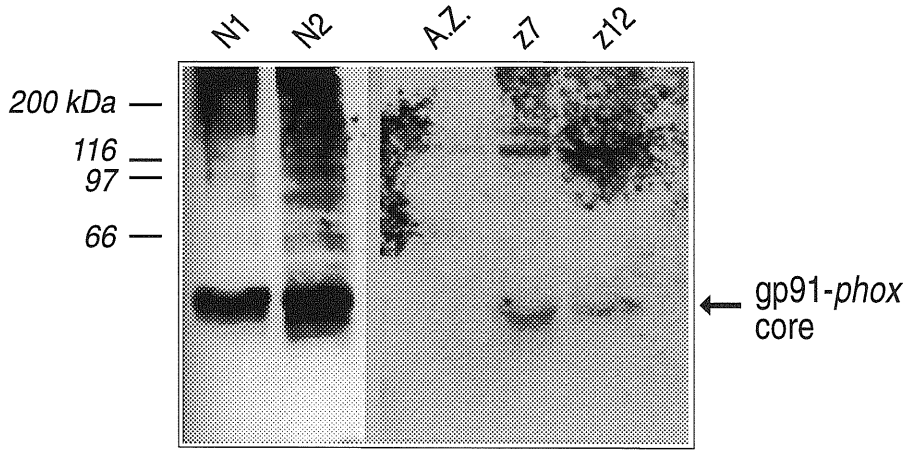


Figure 19. Western blotting of transduced cell clones

Cellular membrane fractions obtained from lymphoblastoid cell lines derived from normal individuals N1 (50 µg) and N2 (50 µg), from patient A.Z.(90 µg), and from transduced clones derived from patient A.Z., z7 (90 µg) and z12 (90 µg), were digested with endoglycosidase F to obtain the core polypeptides, resolved by SDS-PAGE, blotted, and decorated with the monoclonal antibody MoAb 48 against gp91-*phox*.

3. Functional reconstitution of NADPH oxidase activity in transduced B-cell lines.

To assess the restoration of the oxidase enzymatic function in the reconstituted cell lines we used the above described highly sensitive chemiluminescent assay that records the instantaneous level of H_2O_2 formed by the dismutation of O_2^-. We compared the oxidase activity of normal B cell lines before and after stimulation with phorbol myristate acetate (PMA) (figure 16, panel A and B) with that of the transduced and untransduced A.G. and A.Z. clones. As already reported above, the untransduced patient clones were absolutely unable to produce superoxide upon PMA stimulation, while different normal cell lines show a considerable variation of the rate of superoxide production.

It is clear from the data reported in figure 16 that A.G. and A.Z./pBabegp-91*phox* clones show a partial restoration of the oxidase activity. Here again a wide variation in the amount of superoxide generated by the different clones is observed with values that range from 2% to 30% of the most active of the reference normal B-cell lines (figure 16, panel C and D). It should be observed , however, that these values are equal or even higher than those detected in B-cell lines from some individuals.

DISCUSSION

1. Characterization of the molecular defect in three patients with X-CGD

The gp91-*phox* coding sequence of three cases of CGD with $X91^0$ phenotype have been investigated. Sequence analysis of the gp91-*phox* cDNA of these patients revealed the presence of not previously described different point mutations caused by single nucleotide insertions or deletions. The analysis of the nucleotide sequence surrounding the sites of these mutations reveals the presence of elements of interest such as short palindromes and repeated sequences. In patient A.Z., the deletion of the A at position 1330 of the cDNA occurs within a short palindrome AGATCT (the deleted nucleotide is underlined) that overlaps the directly repeated sequence TCTACT (AGATCTACT/TCTACT, with the direct repeat typed in bold letters). The G deleted at base 55 in patient N.B. is positioned within the sequence TTTGTCATTGTA (the deleted nucleotide is underlined), showing a repetition of the sequence TTGT (bold letters). The single base inserted between bases 403 and 404 in patient A.G. also occurs in a region that shows, from base 381 to 407, both TG repeats and the presence of two imperfect direct repeats (TGTGGAATG/G/TGTGTGAATGCCCTGAGT.)

Elements such direct and inverted repeats are commonly found in the vicinity of several human gene deletions (149) and single base insertions (151). Mechanisms of mutagenesis have been proposed that address the role of endogenous sequence environment in promoting such kind of errors (149), (22). For example, in p47-*phox* deficiency, the most frequent abnormality described is a GT deletion that occurs within a repeated sequence in a region that flanks inverted repeats and α-polymerase pause sites (295). All these considerations lead to the

conclusion that the mutations detected in the X-CGD patients analyzed in this thesis are also likely to have been induced by the peculiar base compositions present at those sites. Accordingly, also in another described patient (234), a T deletion occurs at nucleotide 59 within the same TTGT repeat bearing the G deletion in patient N.B.

The very heterogeneous nature and localization of the lesions leading to X-linked CGD clearly exclude the existence of preferential sites of mutation. Therefore, it is not surprising that all the three mutations detected in this study have not been previously described. Given the variety of mutations detected in these patients, the precise identification of the genetic defect in each proband is absolutely required for the purpose of family screening, carrier identification, prenatal diagnosis and genetic counseling.

The reasons for the disease are intuitive in patients A.G. and N.B., since the detected mutations predict the occurrence of a frameshift and the creation of premature stop codons at aminoacids 133 and 21 respectively. In patient A.Z., the frameshift due to the deletion at nt 1330 predicts a protein that lacks 100 aminoacids near its C terminus. This relative hydrophilic domain resides on the cytoplasmic face of the membrane and includes sites needed for NADPH binding and for functional interaction with cytoplasmic components during the activation of the oxidase (256), (243). Moreover, in this patient the level of gp91-*phox* mRNA is also greatly reduced. The deletion of the A at nt 1330 occurs at position +1 of the 3' splice-site consensus sequence at intron-exon 11 boundary. Due to this deletion, the first nucleotide of exon 11 now is a T, thus creating a splice acceptor sequence less favorably recognized by the spliceosome (259). Therefore, the considerable reduction in mRNA level is possibly a consequence of suboptimal terminal splicing of the transcript with consequent mRNA instability (195), (65). In this regard, it should be

observed that we have not been able to detect any abnormally spliced mRNA species even by PCR amplification.

The competitive RT-PCR-based method developed for the quantitation of gp91-*phox* expression is a highly sensitive and precise technique. In particular, the method represents a powerful tool for monitoring transcription even when only limited amount of samples are available. Possible applications of this technique in the gene therapy field, in addition to gp91-*phox* transcript measurement in peripheral blood granulocytes, are the quantitative analysis of retroviral packaging cell lines to rapidly screen for the best titer.

2. Correction of the genetic defect by retrovirus-mediated gene transfer

We have used EBV transformed B-lymphocytes derived from the patients to test the genetic reconstitution of the gp91-*phox* defect by retroviral gene transfer. Since EBV-transformed lymphoblasts from CGD patients mimic the oxidase defect of phagocytic cells, they represent a very convenient in *vitro* model system for the disease (294). Success in gene transfer experiments in EBV B-cells has been already achieved for the deficiencies of both the autosomic p47-*phox* (50), (281), (42), (295) and p22-*phox* (168), and for the deficiency of X-linked gp91-*phox* (221) using retroviral or episomal vectors.

It should be considered, however, that EBV-transformed B cell lines show a great variability in the efficiency of O_2^- production from clone to clone of different individuals (see ref. (295) and data in figure 16). Accordingly, also the rate of transcription of the gp91-*phox* gene can vary, perhaps as a response to minor, uncontrollable modifications of cell culture conditions. Given these considerations, it is not surprising, therefore, that also the response of different clones transduced with the

retroviral vector used in this work display large variability in the efficiency of oxidase function, with peak values in the range of 20-30% of those of the normal cell clone giving the highest value in the set of clones analyzed.

It has been reported that the expression of the transmembrane cytochrome b subunits is rather inefficient as compared to that of the cytosolic components of the oxidase (245) (161), (157). However, the results obtained in this study demonstrate that even a not abundant 91-kDa protein production can be sufficient to assembly a correct cytochrome b_{558} complex and to restore the oxidative burst response to levels that can be of clinical value. In this respect, it is very encouraging to observe that carrier individuals with only 10% of functional phagocytes are perfectly healthy (236). The extent of expression of gp91-*phox* mRNA in the different clones roughly correlates with the intensity of oxidase activity as measured by the functional chemiluminescent assay (see figure 16). However, the production of O_2^- in some clones (for example, in clones z10 and z12) is higher than the one simply predicted by mRNA expression analysis.

Although further data on the utilization of this retroviral vector on hematopoietic precursors are needed for better quantitative evaluation of the restoration of oxidase function, the results obtained confirm that functional correction of the disease can be achieved by this strategy.

3. Perspectives for somatic gene therapy

The data presented in this work, as well as similar conclusions obtained by different laboratories (221), (161), indicate that functional correction of X-linked chronic granulomatous disease can be achieved by virus-mediated gene transfer. Also from the clinical point of view, it

should be considered that the treatment of CGD by gene transfer in hematopoietic precursors has a strong rational basis, since the disease is manifested exclusively on the myelomonocytic cell lineages.

A problem related to hematopietic gene therapy concerns the *in vivo* long-term expression of newly introduced therapeutic gene. It has been shown that in primates bone marrow transduction, considerable problems remain with respect to this problem (287), (29). In addition, it must be mentioned that transcription of DNA sequences for gp91-*phox* has been shown to require *cis* elements and *trans* factors that have not yet been fully elucidated (263). For this reason a genetic cure for X91 CGD patients may be more difficult than for A47 CGD patients.

Although problems concerning the *in vivo* expression of the therapeutic gene and the correction rate achieved with viral vector have yet to be solved, nevertheless gene therapy for CGD patients can be considered as the therapeutic strategy of choice in a not too distant future.

MATERIALS AND METHODS

1. Materials and cell lines

RPMI 1640 was obtained from Gibco BRL Life Technologies LTD (Paisley, Scotland); labeled (^{32}P, ^{35}S) deoxynucleotides were obtained from Amersham International plc (Amersham, UK); Nytran membranes were obtained from Sleicher & Schuell (Keen, NH); TA cloning kit was obtained from Invitrogen Corporation (San Diego, CA); T7 Sequencing kit was obtained from Pharmacia Biotech (Piscataway, NJ); endoglycosidase F was obtained from Boehringer Mannheim (Mannheim, Germany); hygromycin B, polybrene, horseradish peroxidase, phorbol myristate acetate (PMA), luminol, 1,4-piperazinediethanesulfonic acid (Pipes, potassium salt), diisopropylfluorophosphate (DFP) and Ficoll-Hypaque were obtained from Sigma Chemical Co. (St. Louis, MO).

All the oligonucleotides used in this thesis were synthesized by the ICGEB Oligonucleotide Synthesis Service on an Applied Biosystem 380B synthesizer using phosphoramidite chemistry.

The PA317 murine amphotropic retroviral producer cell line, the NIH3T3 murine fibroblast cell line, and the B95-8 marmoset lymphoblastoid cell line were obtained from the American Type Culture Collection (Rockville, MD); the Ψ2 murine fibroblasts ecotropic packaging cell line (170) was kindly provided by Dr. Maria Pia Grossi, University of Ferrara, Italy.

Restriction and modification enzymes were purchased from New England Biolabs (Beverly, MA); *Taq* DNA polymerase was purchased from Perkin-Elmer (Roche Molecular Systems, Branchburg, NJ) .

Monoclonal antibodies MoAb 449 and MoAb 48, directed against gp91-*phox* and p22-*phox* respectively (291), were a kind gift of Dr. Verhoeven, Academic Medical Center, University of Amsterdam, The Netherlands.

2. Patients

Three not previously described male patients (A.G., A.Z., N.B.) of age 15, 21 and 9 respectively affected by X^0 CGD were enrolled in this study. The three patients were affected since the first months of live by serious bacterial infections with slow resolution (in particular, osteomyelitis of long bones, suppurative pneumonia and lymphoadenitis caused by coagulase positive *St. aureus, S. marcescens, Candida*). Laboratory diagnostic criteria were the complete absence of reduction of nitro-blue tetrazolium in the NBT-slide test in granulocytes and the complete absence of oxidase production after incubation with zymosan (at least four tests were performed for each patient). NBT test and oxidase production were normal in the fathers of the three patients, while in the mothers 15-55% of the granulocytes were negative at the NBT test and oxidase production was 32-48% of normal. An affected brother of patient A.G. died due to a granulomatous ventricolitis sustained by *Aspergillus*.

Current clinical features of the three patients are characterized by serious bacterial and fungal infections of different organs.

3. Transformation of B lymphocytes by EBV

3.1 Separation of PBMCs

Ten ml of the heparinized blood samples from normal individuals and affected children were processed within 12 hours from withdrawal. They were mixed with 10 ml of RPMI 1640, layered over 10 ml of Ficoll Hypaque (Sigma, S.Louis, MI, USA) and centrifuged for 40 min. at 1200 rpm. The peripheral blood mononuclear cells (PBMCs) ring was recovered with a Pasteur pipette, transferred to a new tube and washed three times with RPMI 1640.

3.2 Separation of PMN from whole blood

Ten ml of peripheral blood were mixed with 5% dextrane to obtain a 1% dextrane final concentration. After 30 min. sedimentation at room temperature, the upper phase (containing serum and white cells), was diluted 1:1 with RPMI 1640 and centrifuged over Ficoll Hypaque gradient for 40 min. at 1200 rpm. After separation of the mononucleate cells, 5 ml of EDTA 1 mM were added to the pellet containing granulocytes and contaminating erythrocytes, in order to destroy erythrocytes. After a further addition of 5 ml of NaCl 1.8% to reconstitute osmolarity, granulocytes were pelleted by a 5 min. centrifugation at 1000 rpm. The recovered granulocytes were divided in about $5x10^6$ cell aliquots, and 1 ml of 4 M guanidinium thiocynate was added to each aliquot for subsequent RNA extraction. The samples were immediately frozen and kept at -80°C.

3.3 Culture of the lymphoblastoid starter cell lines B95-8 and purification of EBV

PPLO-free cells of the EBV-infected marmoset cell line B95-8 were grown in RPMI 1640 containing 50 µg/ml gentamicin, 10% fetal calf serum and 2 mM L-glutamine. When the cells reached a concentration of about 10^6/ml, the EBV-containing supernatant was harvested by centrifugation at low speed (1200 rpm) to remove cells and debris. The supernatant was then passed twice through a 0,45 µM membrane filter (Millex, Millipore) to further remove cells and debris. This virus preparation was kept at 4°C for several days. Just before use, this supernatant was diluted 1:1 with fresh medium containing 50 µg/ml gentamicin, 10% fetal calf serum and 2 mM L-glutamine.

3.4 Establishment of lymphoblastoid cultures

Total PBMCs were resuspended in the supernatant of the B95-8 cell line (diluted 1:1 with fresh medium), at a cell concentration of $2x10^6$/ml. Two ml of culture was established in sterile flask, pH adjusted to 6.8 by CO_2 addition, and culture was incubated upright at 37°C. Half of the virus-containing medium was replaced at the latest 24 hours after starting of the culture by addition of RPMI 1640 with 20% fetal calf serum, 2 mM L-glutamine, 50 µg/ml gentamicin (final concentration) and 2 µg/ml (final concentration) of cyclosporin A (Sandimmun, Sandoz). The medium was then refreshed once a week by removing half of the supernatant and replacing it by fresh medium containing 1 µg/ml of cyclosporin A.

4. DNA extraction from lymphoblastoid cells and Southern blot analysis of genomic DNA

Established B lymphoblastoid cells from the patients and from normal individuals were grown in RPMI 1640 containing with 50 µg/ml gentamicin, 10% fetal calf serum and 2 mM L-glutamine. 10^8-10^9 cells were centrifuged (10 min. at 1000 rpm), washed twice with PBS, and the pellet resuspended in 2 ml lysis buffer B (SDS 1%, EDTA 0.5 M, proteinase K 1mg/ml). After overnight incubation at 55°C, one volume of phenol: chloroform:isoamylic alcohol (25:24:1) was added. The tube was inverted gently and centrifuged 5 min. at 3000 rpm at room temperature. The supernatant was then transferred to a new tube, extracted with ether, and dialyzed three times against 2-3 liters of TE (Tris 10 mM, EDTA 1 mM). The amount and the quality of the DNA extracted was analyzed by spectrophotometric analysis.

Twenty µg of DNA extracted from established lymphoblastoid cells of healthy donors and of affected individuals were digested to completion with various restriction enzymes (*Eco RI, Hind III, Nsi I,* for the analysis of gp91-*phox* gene, and *Kpn I* and *XbaI* for the analysis of integrated provirus in transduced cell clones). Southern blot analysis was performed using 0.8% agarose gels and 0.45 µm pore-size nylon membranes according to standard methods (249).

As probe for gp91-*phox* gene we utilized an agarose gel purified *Pst I-Sac I* fragment of gp91-*phox* cDNA, containing most of the coding region (1.6 kb), derived from pBsII-KS vector (Stratagene, La Jolla, CA, USA); for the pBabe Higro/gp91-*phox* proviral DNA we used a *Cla I-Hind III* fragment from plasmid pBabeHygro encompassing the hygromycin gene. All the probes were labeled with (α^{32}-P)-dCTP by the random priming

technique with a Promega kit (Madison, WI, USA) according to the manufacturer's instruction.

5. RNA extraction and Northern blot analysis

Total cellular RNA was extracted from $5x10^6$ PMN, $3x10^6$ HL60 and from $2x10^6$ B cells according to the method of Chomczynski and Sacchi (46). For the Northern blotting experiments, samples of 20 µg were resolved in 1% agarose gels in the presence of formaldehyde, and transferred to a 0.45 µm pore-size nylon membranes and hybridized as described (249), with the following modifications: filters were washed two times at 68°C for 15 min. in 1xSSC, 0.1% SDS and once at 68°C for 15 min. in 0.5xSSC, 0.1% SDS. As probes we used the same probes utilized for Southern blot hybridization and a β-actin probe, used as a control for integrity of the RNA. This probe was a 226 bp PCR amplification product obtained by amplification with the BA1 and BA4 primers, (see below). The probe used for c-*myc* detection was a 1500 bp EcoRI DNA fragment, including the first and second exon.

6. Quantitative PCR

6.1 Construction of competitor RNA

Competitor RNA fragments were constructed for the quantitation of the β-actin mRNA (to be used as internal standard for total RNA quantification) and for the gp91-*phox* mRNA from normal individuals and CGD patients by an application of the recombinant PCR methodology (115), (116). The sequence of the oligonucleotides utilized in this work and the amplification conditions are reported in table 10. Oligos CGD8-CGD2

are complementary to the gp91-*phox* transcript (1932-1913 bp and 311-292 bp respectively); oligo BA4 is complementary to the β-actin transcript (311-292 bp). They were utilized as primers for reverse transcription. The cDNA obtained was amplified with primers CGD3T7/CGD8 and CGD1T7/CGD2 for gp91-*phox* and BA1T7/BA4 for β-actin. Primers CGD3T7-CGD1T7 contain a region homologous to gp91-*phox* transcript (1588-1607 bp and 31-51 bp respectively) at their 3' ends; primer BA4T7 contains a region homologous to the β-actin transcript (86-105 bp), at its 3' end (table 10 plain typed nucleotides). At their 5' ends they have a sequence recognized by T7 RNA polymerase (bold typed nucleotides) and the recognition sequence for restriction enzyme *BamHI* (italic typed nucleotides). Forty PCR cycles were performed according to the conditions showed in table 10

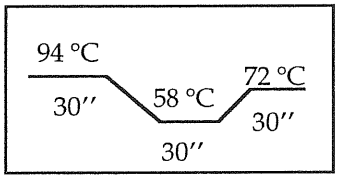
Two further oligonucleotides were synthesized for two amplification sets (CGD3+, CGD8+, CGD1+, CGD2+ for gp91-*phox* amplifications and BA1+, BA4+ for β-actin amplification). These primers have 20 nt at their 3' end identical to contiguous sequences on the upper and lower strands of the amplification products (CGD3+: 1661-1680 bp; CGD8+: 1660-1641 bp; CGD1+: 121-140 bp; CGD2+: 120-101 bp; BA1+: 165-184 bp; BA4+: 164-145 bp.) and 20 nt at their 5' ends complementary each other and unrelated to the amplification products (table 10, bold typed nucleotides: this sequence contains three restriction sites for the restriction enzymes *Pst I, Bam HI* and *Hinc II*).

Two separate amplification were carried out for both gp91-*phox* and β-actin sets of oligonucleotides, with one external primer and the internal primer on the opposite strand (primer CGD3T7 plus primer CGD8+; CGD8 plus CGD3+; CGD1T7 plus CGD2+; CGD2 plus CGD1+ for gp91-*phox* and BA1T7 plus BA4+; BA4 plus BA1+ for β-actin). These amplification products, which contain a single overlapping region of 20 bp, were resolved on a 8% polyacrylamide gel and stained with EtBr. For each of the

gp91-phox

Product size 345 (+20) bp

PCR cycle



Primer	Sequence
CGD3/T7	5′-*CGGGATCC***GGATCCTAATACGACTCACTATAGGGAGA**AGTCAACACCCTAATACCAG-3′
CGD3	5′-AGTCAACACCCTAATACCAG-3′
CGD8	5′-GTAAAAGTGCTCTCAAAACC-3′

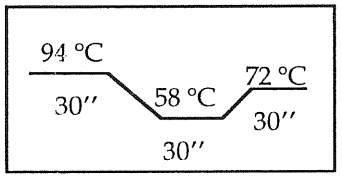
Construction of competitor

Primer	Sequence
CGD3+	5′-**ACCTGCAGGGATCCGTCGAC**AAAGCATCTCCAACTCTGAG-3′
CGD8+	5′-**GTCGACGGATCCCTGCAGGT**GTTTACTCAGGGTTTCAGCC-3′

gp91-phox

Product size 280 (+20) bp

PCR cycle



Primer	Sequence
CGD1/T7	5′-*CGGGATCC***GGATCCTAATACGACTCACTATAGGGAGA**TGAATGAGGGGCTCTCCATT-3′
CGD1	5′-TGAATGAGGGGCTCTCCATT-3′
CGD2	5′-GTGAGATTCCTGTCCAGTTG-3′

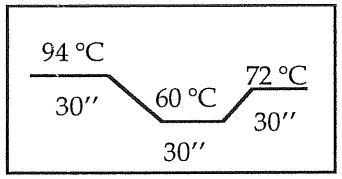
Construction of competitor

Primer	Sequence
CGD1+	5′-**ACCTGCAGGGATCCGTCGAC**CCACCTAAGTTCTTTTACAC-3′
CGD2+	5′-**GTCGACGGATCCCTGCAGGT**AATATCATAAACCCGGTAAT-3′

β-actin

Product size 226 (+20) bp

PCR cycle



Primer	Sequence
BA1/T7	5′-*CGGGATCC***GGATCCTAATACGACTCACTATAGGGAGA**CATGTGCAAGGCCGGCTTCG-3′
BA1	5′-CATGTGCAAGGCCGGCTTCG-3′
BA4	5′-GAAGGTGTGGTGCCAGATTT-3′

Construction of competitor

Primer	Sequence
BA1+	5′-**ACCTGCAGGGATCCGTCGAC**GGCGTGATGGTGGGCATGGG-3′
BA4+	5′-**GTCGACGGATCCCTGCAGGT**CTGGTGCCTGGGGCGCCCCA-3′

Table10. Nucleotide sequences of the oligonucleotides utilized for competitive RT-PCR, and PCR amplification profiles.

three pair of half-amplification, the corresponding bands were touched with the tip of a needle and subsequently soaked in a single test tube containing 50 µl of distilled water. Five µl from each tube were included in a standard 80 µl PCR amplification mixture containing only the two outside primers (CGD3T7 plus CGD8; CGD1T7 plus CGD2; BA1T7 plus BA4). In order to allow the formation of an heteroduplex product annealed at the complementary sequence with 5' protruding ends (the only product which could be further extended and amplified), the reaction was denatured at 94 °C for 1 min., and then the temperature was slowly lowered to 50 °C within 10 min.. After further 2 min. at 50 °C, the reaction was incubated for 5 min. at 72 °C for extension of the annealed products and then amplified using the following PCR cycle profile: the first 5 cycles: 94 °C for 1 min., 37 °C for 30 sec. and 72 °C for 30 sec.; cycles 6 to 10: 94 °C for 1 min., 42 °C for 30 sec. and 72 °C for 30 sec.; cycles 11 to 30: 94 °C for 1 min., 55 °C for 30 sec. and 72 °C for 30 sec.

The amplification products obtained by this recombinant PCR technique has exactly the same sequence as the cDNA of the starting transcript except for the 20 bp insertion in the middle and the addition of a tail with the recognition sequence of T7 RNA polymerase.

RNA competitors were directly obtained from these amplification products (without need for cloning) by in vitro transcription with T7 RNA polymerase using an in vitro kit from Promega (Madison, WI, USA) according to manufacturer's instructions. Forty ng of DNA amplification product were used as template for the reaction.

6.2 Quantification of competitor

Quantification of competitive templates was directly obtained by evaluating the amount of incorporated (^{32}P)UTP in the in vitro transcription reaction as follows. Two µl of (^{32}P)UTP (Amersham, U.K.; 3000 Ci/mmole; 10 mCi/ml) were included in the reaction, corresponding to $2.07x10^7$ cpm, as experimentally evaluated by Cerenkov counting in a beta-counter.

After the transcription reaction, template DNA was removed either by DNase I digestion or by resolution of newly synthesized RNA by denaturing gel electrophoresis (249) and elution from the gel.

An aliquot of the purified competitor RNA preparation was counted and its concentration was evaluated from the final specific activity of the labeled UTP and the number of nucleotides incorporated/molecule.

6.3 Quantitation of gp91-phox and β-actin mRNA in HL60 cells and in clinicals samples

RNA extracted from HL60 cells, granulocytes and lymphoblastoid B-cell lines of normal individuals and CGD patients was submitted to competitive RT-PCR experiments with the β-actin and the two gp91-*phox* competitors RNAs. Four µl of competitor RNA, containing different absolute amounts of molecules (from 10^1 to 10^7 molecules for gp91-*phox* and from 10^7 to 10^9 molecules for β-actin) were mixed to 1 µl of the RNA samples (corresponding to 1/30 of the total RNA extracted from $5x10^6$ granulocytes or immortalized lymphocytes, and retrotranscribed in 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 20 U RNasin (Promega, Madison, WI), 1 mM each dNTP, 20 pmoles of primer and 200

U MuLV reverse transcriptase (BRL, Gaithersburg, MD) (final reaction volume: 20 µl). RNA and primers were annealed in reaction buffer and RNasin for 10 minutes at 65°C and then cooled on ice before addition of nucleotides and enzyme. The reaction mixture was overlaid with 60 µl of mineral oil (Sigma, St. Louis, MO) and allowed to proceed for 45 min. at 42°C. The reaction was stopped by heating at 95°C for 5 minutes.

Sixty µl of a solution containing 250 mM each dNTP, 10 pmoles of the primer used for cDNA synthesis, 30 pmoles of the other primer, 2.5 U of Taq polymerase (Amplitaq, Perkin Elmer Cetus, Norwalk, CT), 50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin were directly added to the 20 µl used for cDNA synthesis. Reaction mixture (80 µl) were subjected to 40 cycles of amplification in a programmable thermal cycler (Perkin Elmer Cetus, Norwalk, CT) using the profiles reported in table 10. Amplification products were resolved on a 8% polyacrylamide gel, stained with ethidium bromide, photographed. The intensity of the bands was determined by densitometric scanning.

7. PCR amplification, cloning and sequencing

The coding region of the gp91-*phox* mRNA was reverse transcribed from 1 µg of total RNA extracted from B-lymphoblasts cell lines from the patients and amplified by PCR using the three top primer pairs reported in table 11. Three overlapping fragments were obtained for each patient. Conditions for reverse transcription (using the antisense primer) were the following: 10 min. annealing at 65°C, 1 hour extension at 37°C, followed by 5 min. at 95°C. Conditions for PCR amplification were: 30 sec. at 94°C, 30 sec. at 56°C, 1 min. at 72°C, followed by 5 min. of final extension at 72°C.

To analyze the very 5' portion that encompasses the initiator ATG codon, genomic DNA was amplified using primers in the 5' untranslated and in first intron regions respectively (primers CGD 11 and CGD 12 in table 11). Polymerase chain reaction was performed in a Perkin-Elmer thermal cycler according to standard procedures.

The amplified fragments were eluted from polyacrylamide gels and directly cloned in the TA vector of Invitrogen. Sequence analysis were performed on plasmid DNA extracted from individual bacterial clones by the dideoxynucleotide chain termination method using a DNA sequencing kit (Pharmacia) based on the utilization of T7 DNA polymerase and ($a^{35}S$)- dATP. Sequence data were obtained for both strands of the inserts by extension of the universal and reverse primers of the vector.

8. Cellular membrane preparation and Western blot analysis

Lymphoblastoid cell lines membranes, from the three patients and from normal controls, were prepared from 10^8 cells.

After washing in PBS cells were resuspended in 1 ml of 6% (w/v) sucrose in 10 mM Pipes (K^+) pH 7.0, and 1 µl of leupeptin (10 µg/µl) and 1 µl DFP (1M) were added. After 15 minutes on ice, cell were lysed by two cycles of sonication of 7 and 5 seconds respectively. Sonicated cells were centrifuged at 2000 rpm for 3 minutes at 4°C. Five hundred µl of 34% sucrose-2 mM EDTA were then added to the supernatant which was then loaded on a sucrose density gradient. The gradient was prepared by layering the following sucrose solutions (w/v, in Pipes pH 7.0): 55% (0.5 ml), 43 % (4.0 ml), 34 % (4.5 ml) and 15 % (1.3 ml). The layers were allowed to diffuse for 3 hours at room temperature to eliminate concentration discontinuities. After centrifugation at 280,000 g (r max) for 2 hours, at 4°C,

the following fractions were collected: cytosol (top of the gradient), band 1, band 2 and band 3. These were diluted with cold 9% sucrose and centrifuged at 230,000 g (r max) for 20 min., at 4°C. After centrifugation, each fraction was resuspended in 100 µl 9% sucrose in 10 mM Pipes (K^+) pH 7.0 and frozen at -80 °C. Protein concentration was determined using a commercial version of the Bradford assay (Bio Rad Protein Assay).

In samples analyzed for the presence of the gp91-*phox* polypeptide, N-linked oligosaccharides were removed by endoglycosidase F according to the manufacturer's recommendation. SDS polyacrylamide gel electrophoresis followed by immunoblotting with MoAb 449 and MoAb 48, directed against gp91-*phox* and p22-*phox*, respectively, was performed as described by Verhoeven (291).

9. Construction of recombinant retroviral vector and establishment of murine producer cell lines

A plasmid containing most of the coding portion of the gp91-*phox* cDNA was obtained starting from plasmid pBsII-KS (kindly provided by Dr M.C. Dinauer), which contains a 1.72 kb *Pst* I-*Sac* I fragment of the cDNA. The missing 5' end of the cDNA coding region (~170 bp) was obtained by cloning of a *Bam HI -Pst* I fragment obtained by PCR amplification from normal granulocyte RNA using primers GP915' and GP913' whose sequence is reported in table 11. The former overlaps the ATG initiation codon and contains a *Bam HI-Kpn* I- *Xho* I polylinker at the 5' end. The nucleotide sequence of the construct was determined. From this construct, the full 1.7 kb coding region of the gp91-*phox* cDNA was excised by *Xho* I digestion and cloned into the compatible *Sal* I site of the retroviral vector pBabeHygro (187) to obtain pBabeHygro/gp91-*phox*. This plasmid was introduced into the ecotropic packaging cell line Ψ2

			cDNA
CGD 1:	20nt	TGAATGAGGGGCTCTCCATT	32-51
CGD B:	26nt	GTACAATTCGTTCAGCTCCATGGATG	675-700
CGD A:	25nt	CCGGAGGTCTTACTTTGAAGTCTTT	606-630
CGD DII:	20nt	GCAAACCACTCAAAGGCATG	1360-1379
CGD C:	25nt	GGTGATGTTAGTGGGAGCAGGGATT	1224-1248
CGD 8:	20nt	GTAAAAGTGCTCTCAAAACC	1913-1932
			genomic map
CGD 11:	20nt	GCATAGTATAGAAGAAAGGC	-36/-16
CGD 12:	18nt	TGGTACTTACAATGACAA	3-20
GP915':	40nt	TTAGGATCCGGTACCTCGAGCCACCATGGGGAACTGGGCT	
GP913':	20nt	ATGCAGTTGAAATTCAGGCA	

Table 11.

Primers used for cDNA amplification, cloning and sequencing. Numbering is referred to the transcription star site of gp91-*phox* mRNA.

using the calcium phosphate co-precipitation method and selection was applied by addition of 200 U/ml of hygromycin B. The resistant colonies were pooled and the supernatant, containing ecotropic recombinant retrovirus, was used to infect PA317 amphotropic cells in the presence of 8 μg/ml polybrene.

The transduced PA317 cells were then replated and selected with 200 U/ml of hygromycin B. Resistant colonies were isolated by ring cloning, expanded and titered for their production of recombinant retrovirus by colony assay on NIH3T3 cells (181). The clone producing the highest titer (~1 x 10^4 cfu/ml) was used as a source of virus for transduction of B-lymphoblastoid cell lines of the patients.

10. Transduction of EBV B-lymphoblastoid cell lines

Subconfluent PA317 cells were cocultured with 5 x 10^5 cells/ml from EBV B-lymphoblasts of patients A.Z. and A.G. in the presence of 6 μg/ml of polybrene. After overnight incubation, non-adherent cells were collected by centrifugation and resuspended in fresh RPMI 1640 medium supplemented with 20% FCS. Hygromycin B selection (200 U/ml) was started 48 hours later and continued for 4 weeks. Surviving cells formed typical cluster aggregates that were isolated and grown separately. These clones were used for DNA and RNA extraction and tested for superoxide production.

11. Superoxide production assay

Luminol assay for testing superoxide production was performed according to Porter et al. (220). Briefly, B-lymphoblasts were washed and resuspended in 1 ml of pre-warmed (37°C) test buffer (130 mM NaCl, 4.6

mM KCl, 1.1 mM KH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$, buffered at pH 7.4 with NaOH) at a final concentration of 10^6 cells/ml in the presence of 9 U/ml of horseradish peroxidase. Chemiluminescence was monitored using a liquid scintillation instrument. Luminol was added just before the first measurement (time 0 figure 16) at a final concentration of 9 µM. Cells were stimulated 10 minutes before the second measurement (time 10') by the addition of PMA, used at a final concentration of 200 ng/ml. Subsequent readings were taken at 10 minute intervals until a maximum of 80 minutes.

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