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Functional Reconstitution of Oxidase Activity in X-linked Chronic Granulomatous Disease by Retrovirus-Mediated Gene Transfer

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Candidate: Sabrina Tafuro
Supervisors: Dr. Mauro Giacca
Dr. Lorena Zentilin

SISSA - SCUOLA INTERNAZIONALE SUPERIORE DI STUDI AVANZATI
TRIESTE
Strada Costiera 11

TRIESTE

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Synopsis

In this thesis, I will report on the results of several molecular studies carried out on three patients affected by X-linked Chronic Granulomatous Disease (CGD). CGD is a rare inherited disease that induces a profound predisposition to severe, often fatal infections. Mutations in the gp91-*phox* (*ph* phagocytes; *ox* oxidase) protein, the large subunit of cytochrome b_{558}, lead to an X-linked inherited form of CGD (X-CGD) that accounts for the majority of the cases.

CGD is a very suitable disease for a gene therapy approach, since the molecular defect is functionally relevant only in cells of the myelomonocytic lineage, and bone marrow transplantation has been curative in some cases. Furthermore, it has been reported that carrier individuals with as little as 10% of functional phagocutes are perfectly healthy. These observations suggest that transfer of the genetically correct gene in hematopoietic precursors followed by autologous transplantation should be curative of the disease.

The experimental work presented in this thesis has been divided into three main sections:

i) *Characterization of the genetic defect in the cells from three X-CGD patients.*
Three different point mutations have been detected in three Italian X-CGD patients. All the mutations lead to a shift in the mRNA reading frame predicting the consequent formation of a truncated protein.

ii) *Construction of retroviral vectors for gp91-phox gene transfer and development of a quantitative PCR procedure for retrovirus titering.*
Four different retroviral vectors were constructed, in which transcription of the gp91-*phox* cDNA was driven either by the Moloney murine leukemia virus long terminal repeat (LTR), or by internal promoters (including the herpes simplex virus-thymidine kinase (HSV-TK) and cytomegalovirus immedite early (CMV IE) promoters and an interferon-γ inducible promoter). A method for rapid titering of retroviral vectors was developed based on competitive PCR quantification of proviral DNA molecules formed in target cells at few hours after infection.

iii) *Transduction of the gp91-phox cDNA in myeloid cell lines, lymphoblastoid cell lines derived from X-CGD patients and in hematopoietic progenitors.*
The results obtained upon infection showed that all the four vectors were able to functionally reconstitute oxidase activity in X-CGD cell lines and primary hematopoietic cells. The retroviral construct expressing the therapeutic gene directly from the viral LTR scored as the most effective in directing expression of the gp91-*phox* cDNA. In addition, the Moloney LTR promoter strongly affected the transcription driven by the internal promoters in myeloid cell lines and primary myeloid cells obtained upon differentiation of hematopoietic progenitors.

The results achieved represent a first step in a gene therapy programme aiming at the correction of the genetic defect by *ex vivo* gene transfer into the hematopoietic cells of the patients.

Introduction - part I

1. NADPH OXIDASE AND THE RESPIRATORY BURST OF PHAGOCYTIC CELLS

1.1 Normal microbicidal mechanisms of phagocytic cells

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

Oxygen-independent mechanisms

Several lines of evidence support a role for microbicidal activity that occurs in the absence of oxygen. Firstly, the efficacy of oxygen independent mechanisms can be demonstrated by the bactericidal activity of neutrophils in oxygen-depleted systems (Mandell, 1974). 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 (Weiss et al., 1982). Thirdly, constituents of neutrophil granules have bactericidal capacity.

The antibacterial protein activity have been localized to cytoplasmic granules (Spitznagel and Shafer, 1985) and several antibacterial protein have been described. Table I describes the best known proteins indicating subcellular location, optimal pH activity and susceptible microorganisms.

Oxygen-dependent mechanisms

Two different microbicidal mechanisms of phagocytic cells are dependent on the consumption of oxygen. They are the myeloperoxidase system and the respiratory burst. The respiratory burst is the enzymatic pathway impaired or completely abolished in chronic granulomatous disease patients.

The 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. Stimulated neutrophils release the products of the respiratory burst and the contents of the granules, including MPO, into the

Table I - Bactericidal mechanisms of phagocytic cells

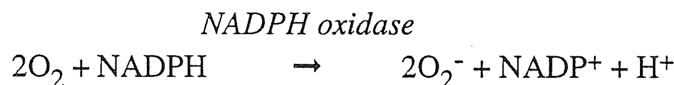
Bactericidal protein	*Subcellular location*	*Optimal pH*	*Susceptible species*
Cation antimicrobial proteins (Mr=37000 and 57000)	Mixed granules	5.6-7.4	Gram-negative bacteria
Bactericidial/permeability-increasing protein	Azurophilic granules	7.0	Gram-negative bacteria
Defensins (Mr<3500)	Azurophilic granules	7.0-8.0	Gram-positive and Gram-negative bacteria, *C. neoformans*, HSV-1
Azurophil-derived bactericidal factor (Mr=29000)	Azurophilic granules	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

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 hypohalous acid and other cytotoxic intermediates. Hypochlorous acid and the monochloramines, the long-lived oxidants derived from HOCl, possess strong cytotoxic effects deriving from their activity against the bacterial electron transport system, from the ablation of the bacterial adenine nucleotide pool, or from oxidation of iron and sulfur centers critical for bacterial viability (Albrich et al., 1981).

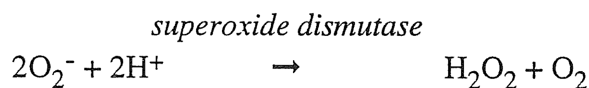
The respiratory burst. During phagocytosis, neutrophils undergo a burst of oxidative metabolism (Babior, 1978). This event begins with a great increase in oxygen consumption as well as in the utilization of glucose via the hexose monophosphate shunt. The process ends with the production of the bactericidal oxygen metabolites superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and singlet oxygen (Halliwell and Gutteridge, 1989). This cyanide-insensitive increase in oxidative metabolic activity is commonly named the "respiratory burst".

The enzymatic complex responsible for the respiratory burst is the NADPH oxidase. This enzyme reduces oxygen univalently using NADPH as electron donor:



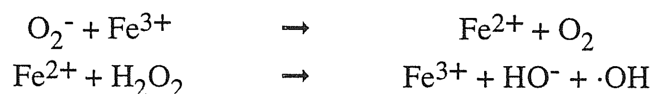
$$2O_2 + NADPH \xrightarrow{\textit{NADPH oxidase}} 2O_2^- + NADP^+ + H^+$$

Most of this O_2^- is thought to react with itself in a dismutation reaction (rapid at physiological pH and more rapidly in the presence of superoxide dismutase) to form the second product of the respiratory burst, hydrogen peroxide:



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

The list of oxygen metabolites generated in the phagocytosis-dependent respiratory burst includes OCl^- and hydroxyl radical ($\cdot OH$); the former 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 $\cdot OH$ production is the interaction between H_2O_2 and iron (Fenton reaction).

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

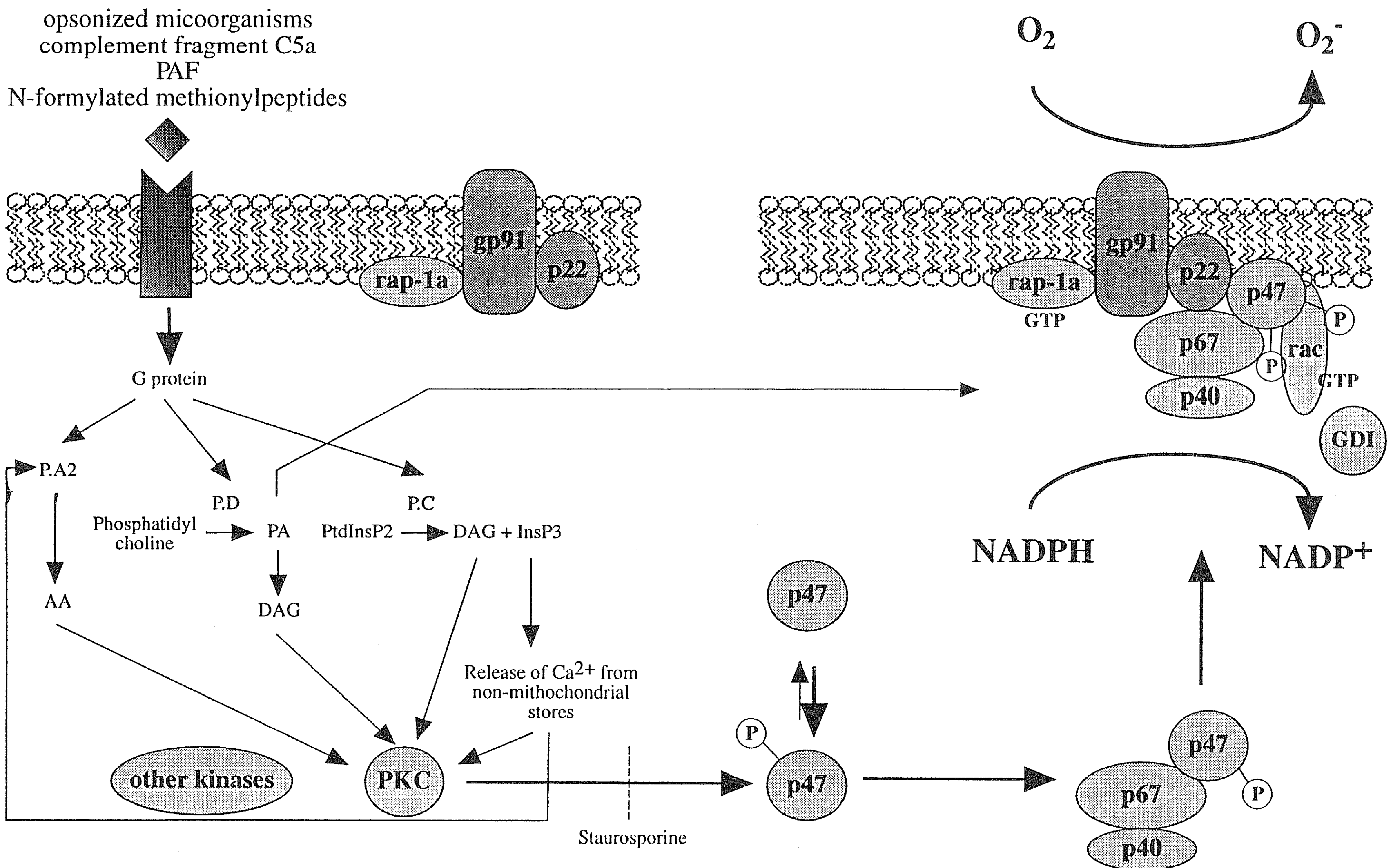


Figure 1. Biochemical mechanisms of activation of NADPH oxidase of phagocytic cells

The formation of all these bactericidal agents depends on the production of O_2^-: thus, 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.

The respiratory burst in phagocytes initiates after ligand binding to surface receptors, with the consequent induction of conformational changes and coupling of these receptors to membrane-bound trimeric GTP-binding proteins. This leads to the production second messengers which in turn activate cytoplasmic protein kinases. These kinases phosphorylate a number of substrates, among which the p47-*phox* protein, with consequent translocation of the p47-*phox*/p67-*phox* complex to the membrane, where it assembles with the cytochrome b_{558} proteins p22-*phox* and gp91-*phox*. The assembled complex is enzymatically active and starts the production of superoxide at the expense of the oxidation of NADPH to $NADP^+$. A schematic representation of the events leading to activation of the NADPH oxidase is presented in Figure 1.

The NADPH oxidase is a multiple-component enzyme, consisting of several subunits. In resting phagocytes, the oxidase complex is inactive and its components are localized in different compartments of the cell. Upon activation, the components are assembled on the cell membrane and the enzyme becomes active. This complex is able to receive electrons from NADPH at the cytosolic side and to donate them to molecular oxygen at the other side, that can be the outside of the cell or within the phagosome compartment.

Two of oxidase subunits are integral membrane proteins that constitute the flavo-heme protein cytochrome b_{558}: they are called gp91-*phox* and p22*phox* due to their molecular mass. The exact stoichiometry of gp91-*phox* to p22-*phox* has been recently elucidated by Huang et al., who showed that the ratio is 1:1 (Wallach and Segal, 1996). The cytochrome b is called b_{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 subunits of the cytochrome are localized in the cytosol of resting phagocytes; among these are p47-*phox*, p67-*phox*, Rac-1 and Rac 2 (GTP-binding proteins), and p40-*phox*. In activated phagocytes, the cytosolic components translocate to the cellular membrane conferring enzymatic activity to cytochrome b_{558}, probably causing a conformational change in the flavocytochrome itself. Five proteins (p22-*phox*, gp91-*phox*, p67-*phox*, p47-*phox*, and one Rac protein) are sufficient to produce superoxide in a cell-free system containing oxygen, GTP, and SDS or arachidonic acid as activator of the oxidase (Rotrosen et al., 1993). In intact cells, additional proteins are probably required for activation and deactivation of NADPH-oxidase (Chanock et al., 1994; Dusi et al., 1996; Morel et al., 1991; Wientjes et al., 1993).

The biochemical events leading to superoxide production , and the molecular features of the NADPH oxidase components are detailed in the following paragraphs.

Due to the fact that all the oxygen metabolites produced by the respiratory burst and released in the phagolysosome are potentially very dangerous for the cell, a number of protective mechanisms have been developed. As toxic oxygen metabolites can damage other circulating cells and adjacent tissues as well as the stimulated phagocyte, it is important that the site of action is concentrated within the phagolysosome. Additionally, several different antioxidant mechanisms are present within the cells, including 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^-. 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. Catalase enzymatically converts H_2O_2 into H_2O and oxygen. Finally, vitamin E (α-tocopherol) reacts with toxic oxygen radicals and preserves cell membranes from oxidative damage. Vitamin C (ascorbic acid) combines with oxygen free radicals to form harmless by-products, and can react with vitamin E radicals to regenerate vitamin E.

1.2 Biochemistry of superoxide production

Early steps in superoxide production

Non stimulated neutrophils consume relatively little O_2. Within few seconds after contact with specific stimuli, the rate of O_2 consumption abruptly increases by a factor of 50-100 (Baldridge and Gerard, 1933). Stimuli that are present at the inflammation sites are represented mostly by opsonized microorganisms, by complement fragment C5a (which is formed upon complement activation after interaction of microorganisms with antibodies), by N-formylated methionylpeptides (that can be released by lysis of dead microorganisms or secreted by bacteria), and by two bioactive lipids produced by activated cells, namely platelet activating factor (PAF) and leukotriene B4 (Snyderman and Uhing, 1988). Other more recently discovered specific stimuli are represented by neutrophil activating proteins (NAP)-1 and 2 (Walz et al., 1989) and melanoma growth-stimulatory activator (Moser et al., 1990).

All these ligands act via distinct surface receptors, and are able to trigger the 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 needed for O_2^- production (Morel et al., 1991). For example, the concentration of the fMet-Leu-Phe peptide necessary to activate chemotaxis is around 10 nM, while that required for stimulation of the respiratory burst is higher than 100 nM.

Also other non physiological stimuli can induce the respiratory burst. Among these, fluoride (Curnutte et al., 1979), 4D-phorbol 12-myristate 13-acetate and some other phorbol

diesters (Robinson et al., 1985), A23187 (Beker et al., 1979) and opsonized zymosan (Burnham et al., 1989). The time required between the interaction of the stimuli with the specific receptor and the superoxide production is less than 5 seconds (Wymann et al., 1987). 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 the neutrophils prior to stimulation. Neutrophils can exist in a resting, activated 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 (Haslett et al., 1989; Johnston and Kitagawa, 1985; Walker et al., 1991).

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 GN (Snyderman et al., 1986) and results in the increase of 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 (Brandt et al., 1985; Ohta et al., 1985; Verghese et al., 1985).

Production of second messengers

Activation of the GN protein in turn results in the activation of three different phospholipases: phospholipase C, phospholipase D and phospholipase A2. They all contribute to the synthesis of second messenger products by an intricate set of reactions ending up with the activation of protein kinase C (PKC) and, possibly, of other not yet well characterized kinases. The signals that determine the differential contribution of each of these phospholipases remain to be determined .

Phospholipase C. Many authors in the past years have demonstrated that neutrophil activators are able to activate phospholipase C through a GN protein (for a review, see; (McPhail et al., 1993)). The consequence of the phospholipase C action is the cleavage of phosphatidylinositol-4,5-bis phosphate (PIP2) with the production of 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) (Traynor et al., 1988). DAG and IP3 are two activators of protein kinase C. DAG acts directly, while IP3 indirectly through a rapid release of Ca^{2+} from non-mitochondrial stores, probably through a specific receptor (Spat et al., 1986). 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 (Lew et al., 1984). A rise in the free cytosolic Ca^{2+} always precedes the onset of the respiratory burst (von Tscharner et al., 1986).

Phospholipase D. DAG can be formed not only by the phospholipase C pathway, but also by an indirect pathway involving phospholipase D. Phosphatidylcholine (PC) is hydrolyzed by phospholipase D to phosphatidic acid (PA) which in turn is degraded by a

phosphatidic acid hydrolase to DAG. Several lines of evidence suggest that also this pathway contributes to superoxide production in myeloid cells (Bowman et al., 1993). Additionally, also PA is directly able to activate NADPH oxidase independently of PKC, acting at a site downstream of PKC activation (Miyahara et al., 1988).

Phospholipase A2. The activity of phospholipase A2 is upregulated during phagocyte oxidase activation, as it can be estimated by the production of fatty acids and, in particular, of arachidonic acid (AA). Furthermore, it has been demonstrated that a variety of phospholipase A2 inhibitors are able to turn off the O_2- generation system (Henderson et al., 1989). In stimulated neutrophils, activation of the phospholipase A2 appears to be under the control of Ca^{2+} concentration and of a G-protein; the rise in cytosolic Ca^{2+} may be subsequent to phospholipase C activation, via the effect of IP3.

In conclusion, DAG, AA and IP3, which are formed as a consequence of the activation of these three phospholypases, are all activators of protein-kinase C. The first two compounds act directly on the kinase, while IP3 acts indirectly through a rapid release of Ca^{2+} from non-mitochondrial stores.

Protein kinases involved in respiratory burst activation

Protein-kinase C (PKC) plays a fundamental role in NADPH oxidase activation. This conclusion is inferred by a number of studies which in the past showed that the respiratory burst is blocked by inhibitors of PKC activation (Wilson et al., 1986). p47-*phox* is a sustrate for PKC in vitro (Kramer et al., 1988; Uhlinger and Perry, 1992), and in activated neutrophils many of the phosphorilated sites of the protein are located in consensus sequences for protein kinase C (el Benna et al., 1994).

The phosphorylation of p47-*phox* is the essential step for the migration of the cytoplasmic components of the enzyme to the cell membrane (Rotrosen and Leto, 1990). PKC exists in multiple isoenzymes, but only the a and b isoforms are present in human neutrophils; the a isoform is largely predominant (Majumdar et al., 1991).

Also other still uncharacterized protein kinases are 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 (Ferrel and Martin, 1989). These data are in favor 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.

Translocation of p47-phox, p67-phox and p40-phox

As reported above, in PMA stimulated neutrophils the phosphorylation of p47-*phox* seems to play a fundamental role in activation of respiratory burst. It has been suggested that phosphorylation occurs in two subsequent steps (el Benna et al., 1994; Knaus et al., 1992;

Rotrosen and Leto, 1990). 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 (el Benna et al., 1994) and in cell-free systems (Caldwell et al., 1988). 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.

In cells lacking p47-*phox*, both p67-*phox* and p40-*phox* are not translocated to the membrane (Dusi et al., 1996); on the contrary, in neutrophils lacking p67-*phox*, p47-*phox* is translocated while p40-*phox* is not; additionally, the latter protein is present in reduced amounts. From these observation, it can be inferred that p67-*phox* is required for p40-*phox* stability and that translocation of both proteins to the membrane follows that of p47-*phox* (Dusi et al., 1996; Heyworth et al., 1991; Kleinberg et al., 1990). This model is further supported by the fact that, in resting cells, p67-*phox* and p40-*phox* are associated in the cytosol (Tsunawaki et al., 1994).

Continuous translocation of the cytoplasmic components of the oxidase is necessary to maintain the enzyme in an active state (Dusi et al., 1993; Tsunawaki et al., 1994): the constant production of superoxide seems to be ensured by the continuous recruitment of new units of NADPH oxidase, which remains only transiently in an activated state.

Formation of active NADPH oxidase complex

Translocation of the cytosolic components of the oxidase to the membrane and interaction of these components with cytochrome b_{558} leads to activation of the enzyme. Synthetic peptides corresponding to the C-terminus region of gp91-*phox* are able to greatly impair both respiratory burst and p47-*phox* phosphorylation in activated neutrophils (DeLeo et al., 1995; Rotrosen et al., 1990). These peptides prevent the interaction between p47-*phox* and gp91-*phox* and block the access of p47-*phox* to a specific kinase responsible for multiphosphorylation (Rotrosen et al., 1990). Interestingly, the same peptides are not able to prevent p67-*phox* binding to the membrane (Malech et al., 1991). From these data, it can be inferred that binding of the cytosolic components to the membrane components of the oxidase occur through p47-*phox* specific binding to gp91-*phox*. A direct binding between p47-*phox* and p22-*phox* has also been demonstrated (DeLeo et al., 1995; Leto et al., 1994; de Mendez et al., 1996).

The complexity of the regulation of the NADPH oxidase activation is further underlined by the discovery of cytosolic GTP-binding factors that are involved in the regulation of p47-*phox*/p67-*phox* translocation (Pick et al., 1989). These factors have been identified as proteins Rac1 (Abo et al., 1991)) and Rac2 (Knaus et al., 1991). Since GTP analogs can modulate the translocation of p47-*phox*/p67-*phox* in a cell-free system (Park and Babior, 1992;

Uhlinger et al., 1993) a role for a GTP-binding protein in modulating the p47-*phox*/p67-*phox* interaction with the membrane components of NADPH oxidase was suggested. The Rac proteins are considered good candidates for this role. In fact, these proteins translocate from the cytoplasm to the plasma membrane of neutrophils upon cell activation (Abo et al., 1994; Dusi et al., 1996; Uhlinger et al., 1993) with a kinetics that slightly precedes the onset of O_2 consumption (Quinn et al., 1993).

Rac1 and Rac2 are present in neutrophils lacking p67-*phox* and p47-*phox*; however, in p67-*phox*-deficient neutrophils Rac1 is not translocated to the membrane even if p47-*phox* is present (Dusi et al., 1996). This observation confirms that Rac1 interacts with p67-*phox* by binding to its NH_2-terminal 199 amino acids (Diekmann et al., 1994). Morover further studies done in a patient with a mutation in the p67-*phox* gene, showed that this mutations disruped the interaction between this protein and Rac 1. The result is the lack of translocation of the cytosolic components p47-phox and p67-phox to the plasma membrane, indicating that the interaction is essential for the translocation itself (Leusen et al. 1996). On the contrary, the absence of both p47-*phox* and p67-*phox* does not affect binding of Rac2 to the membrane, which is instead decreased by the absence of cytochrome b (Heyworth et al., 1994). These data suggest that the interaction of this protein with the oxidase complex occurs through the cytochrome b components.

Recently, in a cell free assay, it has been demonstrated that p67-phox and Rac 1 are the only cytosolic components directly involved in the induction of the electron transport, while the presence of p47-*phox* can optimise the generation of 0_2^-, possibly stabilizing the interaction of the other two with cytochrome b_{558} (Koshkin et al., 1996).

1.3 Components of the superoxide-generating oxidase of phagocytic cells

The genes for the two subunits of cytochrome b_{558}, p22-*phox* and gp91-*phox*, as well as those for p47-*phox* p67-*phox*, and p40-*phox* have been localized, cloned, and characterized. The data concerning the characterization of these genes and the relative mRNA and proteins are summarized in Table II.

Table II. Components of the NADPH oxidase of phagocytic cells

		p22-*phox*	gp91-*phox*	p47-*phox*	p67-*phox*	p40-*phox*
Gene	*Locus*	CYBA	CYBB	NCF1	NCF2	NCF4
	Chrom. location	16q24	Xp21.1	7q11.23	1q25	22q13.1
	Size	8.5 kb	30 kb	17-18 kb	40 kb	18 kb
	Exons	6	13	9	16	10
mRNA	*Size*	0.8	5 kb	1.4 kb	2.4 kb	1.2 kb
Protein	*Amino acids*	195	570	390	526	339
	Mol. mass predicted	20.9 kDa	65 kDa	44.6 kDa	60.9 kDa	/
	Mol. mass SDS-PAGE	22 kDa	76-92 kDa	47 kDa	67 kDa	40 kDa
	pI	10.0	9.7	10	6	/
	Location in resting phagocytes	Membrane	Membrane	Cytoplasm	Cytoplasm	Cytoplasm
	Post-translational modification	Phosphorylated	N-linked carbohydrates phosphorilated	Phosphorylated during oxidase activation		

From Roos D., 1994

gp91-phox

The gp91-*phox* gene has been one of the first genes cloned by reverse genetics and positional cloning in 1986 (Royer-Pokora et al., 1986). The gene encodes for a 571 aminoacid sequence, although the protein has a relative molecular weight of 76,000 to 92,000 kDa due to extensive glycosylation. It is a membrane protein containing several transmembrane domains resulting in the formation of external and cytoplasmic loops; a schematic representation of the protein is shown in Figure 2.

Using anti-peptide antibodies, it has been possible to map the protein segments exposed on the cell surface (Imajoh et al., 1992). Two of such regions, exhibiting high hydrophilicity, were found between residues 150 and 172, close to two possible glycosylation sites (Asn 132 and 148) and between residues 369 and 398.

The region 399-421, immediately after the second hydrophilic region, is hydrophobic and is considered to be a transmembrane segment. The carboxy-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 (Imajoh et al., 1992). 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, 4676 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. The histidines at position 100, 110, 117, 207, and 220 are in the hydrophobic regions and are spaced similarly to the heme-coordinating histidines in other cytochromes (Royer-Pokora et al., 1986).

Further analyses of the C-terminal segment of the protein were conducted with synthetic peptides. The addition of a 7-aa peptide corresponding to aminoacids 559-565 was able to block superoxide production in a cell free system, thus suggesting that this portion of the protein is essential for the enzymatic function (DeLeo et al., 1995; Rotrosen and Leto, 1990). Additionally, the same peptide also inhibited p47-*phox* phosphorylation. The simplest model that can account for both inhibition of oxidase activity and p47-*phox* phosphorylation is the one which assumes that the C-terminus of gp91-*phox* is essential for assembly of the oxidase components.

Other information about gp91-*phox* could be deduced from alignment analysis of the amino acid sequence. Alignment with several flavoproteins revealed significant similarities. In particular, two gp91-*phox* regions (residues 218-223 and 350-360) were similar to the FAD binding site of human glutathione reductase and related enzymes (Eggink et al., 1990). In addition, also the region 335-345 exhibits some similarity to the FAD-binding domain of ferredoxin reductase and similar enzymes (Bredt et al., 1991; Segal et al., 1992). Altogether, these observations indicate that the middle portion of gp91-*phox* is likely to form a FAD-binding domain.

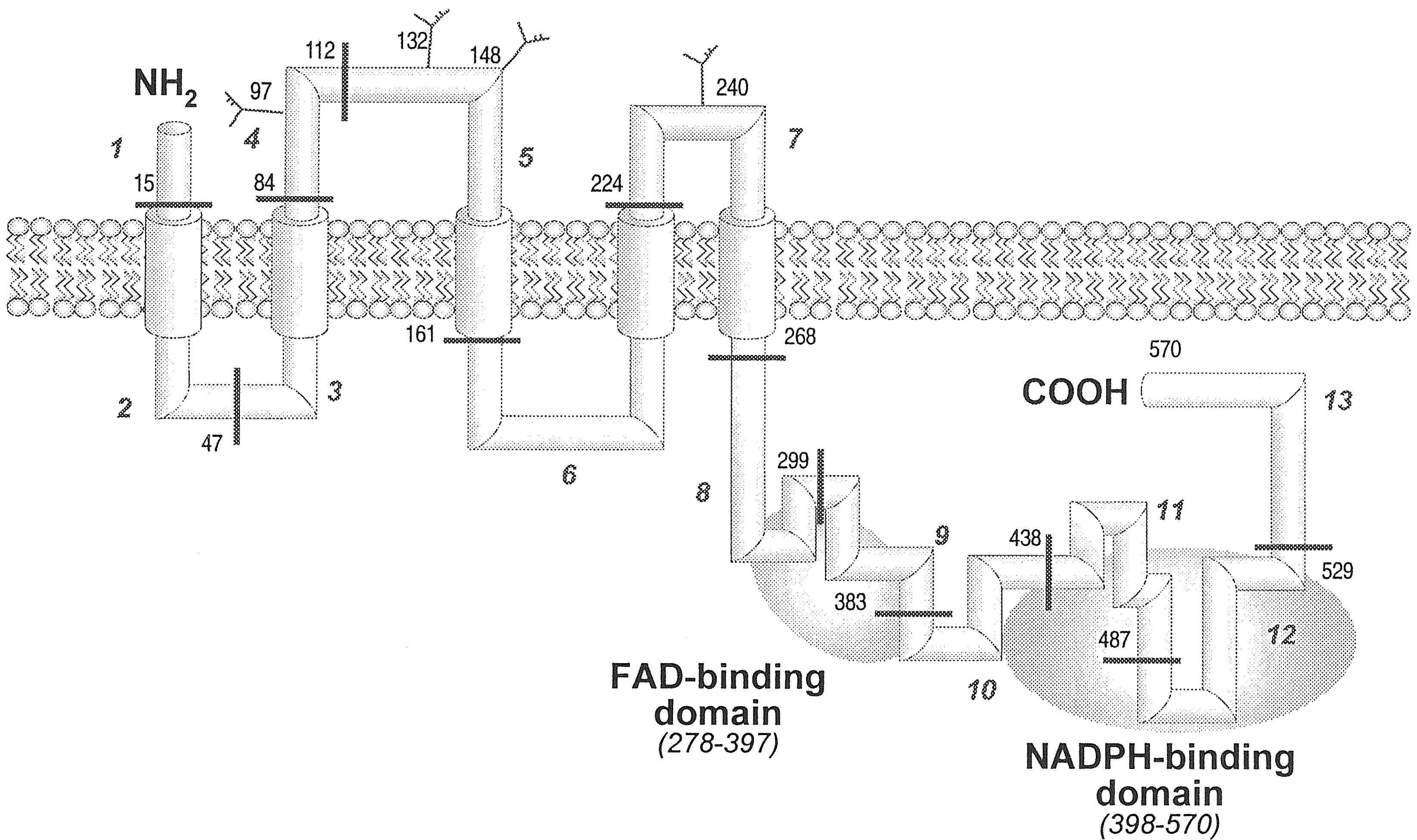


Figure 2. Schematic representation of gp91-phox.
Exons are numbered progressively, intron-exon boundaries are indicated by crossing segments and glicosilated sites in the outer portion of the protein are shown. The FAD-binding domain and NADPH-binding domain of the protein are shown are shadowed areas behing the intracytoplasmic portion.

Other interesting similarities can be found between gp91-*phox* and some NADP(H)-dependent flavoenzymes (Sumimoto et al., 1992; Taylor et al., 1993). In particular, there are two regions in the C-terminal portion of gp91-*phox* (aminoacids 405-423 and 531-546) homologous to the NADPH-binding domains of proteins of the FNR family (Karplus et al., 1991) and one region (441-450) homologous to enzymes closely related to cytochrome P-450 reductase (Vogel and Lumper, 1986).

In conclusion, these data altogether indicate that the N-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 C-terminal portions are involved in FAD binding and NADPH binding respectively (Figure 2). The C-terminal segment seems also to play a critical role in the interaction with the cytosolic components of the oxidase complex. The homologies between the FAD-binding site and glutathione reductase and related enzymes, and between the NADPH-binding site and the FNR proteins, suggest that the gp91-*phox* gene probably has arisen through a fusion of the ancestral genes for these two distinct types of flavoproteins (Karplus et al., 1991).

The gp91-*phox* core protein becomes heavily glycosylated in the cell. In neutrophils, four possible glycosylation sites have been described (Asn 97, 132, 148 and 240). Only N-linked (and no O-linked) oligosaccharides are present on the core protein purified from neutrophils (Harper et al., 1985). The most abundant monosaccharides are represented by N-acetylglucosamine and galactose (Harper et al., 1985).

Various glycosylation patterns can be found in different phagocytic cells type (Kleinberg et al., 1989): 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-differentiated HL-60 cells. However, after complete digestion with endoglycosidase F, the core peptide has the expected molecular weight of 55 kDa in all phagocytic cell types (see also Figure 4). This means that different enzymatic pathways responsible for post-translational glycosylation act in different phagocytic cells.

The N-linked oligosaccharides contribution to superoxide generation is not well understood. However, it is unlikely to play a critical role since a cytochrome b_{558} complex deficient in N-linked oligosaccharides may be functionally competent to mediate the respiratory burst, although at a lower extent (Kleinberg et al., 1989).

p22-phox

This protein is composed of 195 amino acids and is characterized by a proline-rich C-terminus (27% of the 63 C-terminal residues). The primary structure, as it can be deduced from its cDNA sequence (Parkos et al., 1988), suggests that it is a membrane protein. By studying the antibody reactivity of the protein, it was possible to conclude that the N-terminal and C-terminal portions of the protein face to the cytoplasmic side of the membrane (Imajoh et

al., 1992), since these regions are accessible only upon cell permeabilization. The C-terminus (aa. 150-159) has been proposed to be a possible binding site for the SH3 domain of one of the cytosolic proteins (Wientjes et al., 1993).

By aminoacid alignment analysis, structural motifs in common with other heme-containing proteins have been described (Parkos et al., 1988). 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.

Cytochrome b_{558} heme content

The theoretical heme content for a cytochrome b heterodimer of aggregate molecular mass of 113 kDa (91+22 kDa) containing only one heme is of 8.9 nmol/mg of protein (Quinn et al., 1989). However, the measured heme content of the purified cytochrome b is 20-30 nmol/mg of protein depending on the value used for the extinction coefficient (Parkos et al., 1987). 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 (Hurst et al., 1991; Quinn et al., 1992; Cross et al., 1995), who 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; 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 like yeast (Finegold et al., 1996); in chloroplasts and in bacteria the hemes are present in the membrane bilayer and shared between two different polypeptide chains of the cytochrome molecule. 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 is reduced to generate 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 sites are occupied by intrinsic ligands, and the sixth coordination site 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 (Iizuka et al., 1985), resonance Raman (Hurst et al., 1991) and EPR spectroscopy (Miki et al., 1992). 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.

p47-phox

From the p47-*phox* cDNA sequence (Volpp et al., 1989) it is possible to deduce a protein of 373 amino acid with a molecular mass of about 42 kDa. Analysis of the derived amino acid sequence demonstrates a protein having four major structural features of potential interest.

The N-terminal domains contains a glycine residue (Gly-1) that could potentially serve as a site for myristolylation (Volpp et al., 1989). The N-terminal myristolylation is an early event in acylprotein biosynthesis, which is likely to participate in the juxtapositioning of the acylprotein and other components of cellular regulatory circuits (Towler et al., 1988). 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 myristylation could allow protein translation to the membrane during phagocyte activation.

The C-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 (Edelman et al., 1987). Another potential site is represented by Tyr-97. These sites are in accordance with previous reports of a 47 kDa component of the NADPH oxidase that appears to be a substrate for phosphorylation (Caldwell et al., 1988; Rodaway et al., 1990).

Two GTP-binding domains are present in the p47-*phox* protein (residues 262-268 and 151-154), which might be involved in the interaction with the phosphate portion of the GTP molecule (Klinger and Aviram, 1992).
Finally, by comparing the p47-*phox* sequence with all the 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 the SH3 domain of proteins belonging to the *src* superfamily (Leto et al., 1994; Rodaway et al., 1990). Besides the non receptor tyrosine kinases, these domains are found in a number of other cytoplasmic proteins, including p67-*phox*. In p47-phox these domains are implicated in the interaction with a proline-rich region of p22-phox (de Mendez et al., 1996; Leto et al., 1994).

p67-phox

The nucleotide sequence of the p67-*phox* cDNA (Leto et al., 1990) predicts a 526 amino acid protein with a molecular mass of 61 kDa. As mentioned above, structural similarities exist between two segments of the protein (one in the C-terminus - aa 462-482 - and one in the

middle portion - aa 245-293 -) and the SH3 motif of *src* superfamily. In most other proteins where a SH3 domain was found, concomitant homologies to the SH2 and B domains of *src* are also detectable (Rodawy et al., 1989). On the contrary, just the SH3 domain is present in p67-*phox*, p47-*phox*, myosin I and cc-spectrin, suggesting that this region may function independently of the other two regions. By similarity to the function of the homologues region of myosin I (which is also located close to a proline-rich stretch), it has been proposed that this site could serve for direct binding to actin (Leto et al., 1990).

A proline-rich sequence is present in the p67-*phox* protein (aa 224-235), and this region is involved in the interaction with p47-*phox* through one of the two domains of the p47-phox protein (de Mendez et al., 1996).

Analysis of some regions of the p67-*phox* protein have been recently performed using a transfected plasmid containing the mutated p67-*phox* cDNA. In these studies, it was determined that the neither the last 14 aa nor the first 22 aa residues are essential for the O_2-production (Chanock et al., 1996).

p40-phox

More recently, a new cytosolic component of the NADPH oxidase activation complex has been identified and described (Wientjes et al., 1993; Zhan et al., 1996). The amino acids sequence obtained from its cDNA reveals a protein of 339 amino acids. The similarity between p40-*phox* and p47-*phox* is intriguing: the two proteins have 22% identity over 245 amino acids, which increases to 67% over two thirds of the N-terminus portion if conservative substitutions are taken into account.

p40-*phox* also contains a region (aa 175-225) with similarity to an SH3 domain. However, it was shown that the C-terminus of the protein, but not its SH3 domain, is responsible for the tight association of p40-*phox* with p67-*phox* in the cytosol of resting neutrophils (Tsunawaky et a., 1996). All three proteins (p40/p47/p67-*phox*) translate to the plasma membrane upon activation of the oxidase (Dusi et al., 1996; Fuchs et al., 1995). However, it is worth mentioning that the presence of p40-*phox* is not required for activation of the oxidase in a cell-free assay, since its action may be replaced or circumvented by using SDS or arachidonic acid as activators.

Rac1 and Rac2

The third cytosolic component required for the NADPH activation in a cell free system was identified as p21rac1 in guines pig macrophages (Abo et al., 1991) and p21rac2 in human neutropils (Knaus et al., 1991), two low molecular weight guanosine triphosphate (GTP)-binding proteins called belonging to the Ras related GTP binding proteins. This protein family

has a wide tissue range, and in neutrophils both p21rac1 and p21rac2 are present (Dusi et al., 1995). Rac1 and Rac2 are 95% identical to one another in their amino acid sequence.

These proteins functions changing from an inactive state in which they bind guanosine diphosphate (GDP) to an active, GTP bound form, in which they can mediate the activation of the NADPH oxidase. Both Rac1 and Rac2 were purified as complexes with a dissociation inibition factor Rho-GDI (Abo et al, 1991 Kwong et al., 1993), although Rho-GDI is not required for the *in vitro* activation of the oxidase. The GDP-GTP exchange of rac1 and rac2 is regulated by Rho-GDI, which inhibits GDP dissociation and subsequent GTP binding (Abo et al., 1991). In a cell free system, NADPH oxidase activation is inhibited by peptides mapping within the carboxyl terminal domai of Rac1 (Joseph et al., 1994). Anyway this inhibition is not amino acid specific but related to the presence of a polybasic motif, that can been involved in the membrane targeting.

Physiological aspects of NADPH oxidase gene expression regulation

Many cells can produce superoxide in response to specific stimuli. Messenger RNAs for gp91-*phox*, p47-*phox*, p67-*phox* and p40-*phox* can be mostly detected in neutrophils, monocyte/macrophages, EBV-transformed B cells (Morel et al., 1991; Volkman et al., 1984) and tonsillar B lymphocytes (Maly et al., 1989). In particular, the gp91-*phox* mRNA is abundant in neutrophils, perhaps accounting for 0.1% or more of total cellular mRNA. The control of the specificity of gp91-*phox* transcription has not been fully elucidated yet. A fragment of 450 bp of the 5' flanking region of the gene is able to drive transcription of a reporter gene in a subset of phagocytes, but not in all the myelomonocytic cells (Skalnik et al., 1991). Moreover, a region positioned from -160 to -170 upstream of transcription start site is supposed to exert a negative function on gene expression; this site is bound by a CCAAT displacement protein. This region is probably involved in the control of transcriptional specificity of gp91-*phox* in myelomonocytic cells (Lievens et al., 1995; Luo and Skalnik, 1996). Moreover, an element in the gp91-*phox* promoter (from -50 to -57) is required to confer interferon-γ responsiveness (Eklund and Skalnik, 1995). This element interacts with two DNA binding proteins; one of these proteins, named HAF-1 (hematopoietic associated factor), is restricted to hematopoietic cells, while the other is the ubiquitous CCAAT-binding protein CP1, dispensable for the interferon-γ response. The mutations found in the promoter of X-CGD patients described by Newburger et al. (Newburger et al., 1994) specifically abolish the binding of HAF-1, demonstrating the functional significance of this factor for gp91-*phox* promoter activity. Many other transcription factors are involved in the transcriptional regulation of the gp91-*phox* gene, controlling both transcriptional repression and activation (Luo and Skalnik, 1996, Luo and Skalnik 1996)

Among all the cells producing superoxide, phagocytes produce the largest amount. EBV-transformed cells contain the same oxidase complex present in phagocytes, although the production of superoxide is just 1% to 10% of the amount produced by these cells.

Interestingly, of the four structural components of the phagocytes NADPH oxidase, p22-*phox* is the only component transcribed in cells other than phagocytes (Parkos et al., 1988), while the other four structural proteins are expressed in a highly lineagespecific manner. However, although the mRNA is constitutively expressed, non-phagocytic cells contain little stable p22-*phox* protein. Additionally, p40-*phox* is expressed also in cells that do not generate large amounts of superoxide like mast cells and basophils (Zhan et al., 1996). Finally, eosinophils have a specific mechanism for the control of gp91-*phox* expression, which probably acts at the transcriptional level (Kuribayashi et al., 1995).

Still controversial is the presence (and the function) of NADPH oxidase components on the membranes of other cell types such as human fibroblasts (Meier et al., 1991; Meier et al., 1993), in human mesangial cells or glomerular cells (Radeke et al., 1991), and in epithelial cells (Jones et al. 1996). There are increasing evidences that the NADPH oxidase components can also take part in different systems in which superoxide is generated other than the respiratory burst of phagocytes. For example, Henderson et al. showed that gp91-*phox* is the arachidonate-activable H^+ channel of human neutrophils (Henderson et al., 1995), while in pulmonary neuroepithelial bodies (NEB) of the airway mucosa, NAPDH oxidase is used as a O_2 sensor (Wang et al., 1996).

2. CHRONIC GRANULOMATOUS DISEASE (CGD)

Chronic granulomatous disease (CGD) is a rare hematological disorder with an estimated prevalence between 1:250,000 and 1:500,000 (Roos, 1994). A heterogeneous group of genetic defects is the cause of the disease, which presents with recurrent, severe bacterial and fungal infections usually involving the skin, soft tissues, respiratory tract, lymph nodes, liver and spleen (Curnutte and Babior, 1987; Johnson and Newman, 1977). The disease can be inherited in either an X-linked or an autosomal recessive manner. Therapy requires prolonged prophylaxis with antibiotics in order to reduce and control infections, since infectious episodes can sometimes be fatal. The disease affects professional phagocytes (neutrophils, monocytes and eosinophils) that can still ingest microorganisms but are not able to kill them after ingestion. This failure in microbial killing is due to defects in the production of antimicrobial compounds with oxidative properties, such as superoxide anion, hydrogen peroxide and other oxygen derivatives. In particular, CGD is caused by a defect in the production of superoxide anion (O_2) by the NADPH oxidase of phagocytic cells.

2.1 Clinical findings

Clinical presentation

From the clinical standpoint, 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, and same long survivor patients have been described (Schapiro et al., 1991).

CGD patients are represented mostly by children with a history of recurrent infections. This disease should be considered in any individual with recurrent purulent infections caused by fungi or catalase-positive bacteria. These bacteria are the most common pathogens in these patients because catalase prevents the CGD phagocytes from using microbial-generated H_2O_2 for killing micro-organisms.

In general, it has been reported that patients with defects in cytochrome b_{558} have a more severe clinical course than those with defects in cytosolic NADPH oxidase components (Forrest et al., 1988; Weening et al., 1985) (see below). There is no correlation between the amount of superoxide generation and the severity of the disease demonstrating that the clinical course may depend on other microbicidial nonoxidative mechanisms.

The most common clinical findings are summarized in Table III. 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. 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 (Johnson and Newman, 1977).

Skin infections, mainly represented by pyogenic dermatitis, furunculosis and subcutaneous abscesses (Johnson and Newman, 1977; Tauber et al., 1983), are very common and can be prominent in adults with otherwise mild disease (Barriere et al., 1981).

Infections of the lower respiratory tract are also common. Lobar, bronchial or diffuse and generalized pneumonia can occur with high frequency (Donowitz and Mandell, 1983). On the contrary, lung abscesses are not so common. The consequences of repeated infectious episodes are granulomatous infiltration and fibrosis. The oropharynx and gastrointestinal tract are also 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 inflammatory bowel disease. Osteomyelitis has been found in about one third of patients, especially in metacarpals and metatarsal bones (Johnson and Newman, 1977). Disseminated infections with bacteriemia

Table III. Sites of infection in chronic granulomatous disease

Very frequent (>60%)	Moderately frequent	rare
lungs	middle ear	para-nasal sinuses
lymph nodes	bones	urinary tract
skin	peri-anal region	CNS and meninges
	sepsis	pericardium

From: Curnutte, J.T., 1993

and/or meningitis can less frequently occur (17% of 168 cases described (Johnson and Newman, 1977)).

The most frequent bacterial species associated with infection at almost any sites are *S. aureus*, enteric bacteria and *Aspergillus.* In particular, CGD should be considered in any patient presenting with any infection of a parenchymatous organ sustained by *Aspergillus* or *Serratia.* 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.

Pathologic findings

In an acute infection, infected sites show a necrotic inflammatory process associated with suppuration. If the infection has been prolonged, granulomas are formed with multinucleated giant cells, macrophages, lymphocytes, and plasma cells (Tauber et al., 1983). The formation of granulomas appears as a consequence of the prolonged intracellular survival of microorganisms, similar to other chronic infectious disease of bacterial origin such as tubercolosis (Johnston, 1976) The chronic inflammation does not always result from persistent infections, but also from other abnormalities in the inflammatory response in the absence of a respiratory burst (Morgenstern et al., 1997). 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.

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 (Babior and Crowley, 1983; Johnston, 1976). Other tests of immune function are normal (Babior and Crowley, 1983), with the rare exception of abnormal lymphocyte activation or chemotaxis, thought to be due to serum inhibitors (Clark and Klebanoff, 1978).

Diagnosis

In a patient with clinical symptoms suggestive of CGD, the diagnosis has to be confirmed by the failure of the superoxide production by neutrophiles 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) (Weening et al., 1975). Chemiluminescence with luminol or lucigenin is also often used to measure oxidase activity (Wymann et al., 1987) as well as flow cytometric methods (Roesler et al., 1991). The most common clinical test is the NBT (nitro blue tetrazolium) test, which evaluates the reduction of NBT to its purple formazan crystal by superoxide in neutrophils. 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 (see also below). 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 among the different genetic forms of the disease, each of which is determined by defects in one of the major subunits of the NADPH oxidase (gp91-*phox*, p22-*phox*, p47-*phox*, and p67-*phox*) (see Table IV). The gp91-*phox* subtype is characterized by X-linked transmission (X91), while the other ones by autosomal transmission (A22, A47 and A67 respectively). The most common consequence of the genetic defect is the absence or the reduction of the respective protein in the neutrophils of the patients. According to the degree of reduction, different phenotypes can be recognized in the X-linked form: $X91^{o}$ when no protein can be detected; $X91^{-}$ when subnormal amounts of proteins are present and $X91^{+}$ when normal amounts of proteins are present. In the autosomal forms the phenotypes $A22^{o}$, $A22^{-}$ and $A22^{+}$ are also described. All the defects in the p47-*phox* and p67-*phox* genes so far described lead to complete absence of the respective proteins ($A47^{o}$, $A67^{o}$).

Laboratory differentiation between the different subgroups, begins with western blot analysis of neutrophil lysates with antibodies against the four subunits. In case of $A47^{o}$ or $A67^{o}$ CGD, the distinction is easy, because it is determined by the lack of reactivity with the relevant antibodies. In case of A22 or X91 CGD, the distinction can be more difficult, since both subunits are absent in most of the $A22^{o}$ and $X91^{o}$ CGD, and it is complicated by the presence of the + and - variants. When both subunits are undetectable, searching for carriers in the family of the patients by the NBT slide test can help in the distinction between the $A22^{o}$ and $X91^{o}$ forms. The presence of neutrophils with both functional and non-functional NADPH oxidase in obligate heterozygotes (e.g. the mothers of the patients) proves the X-linked nature of the disease, indicating deficiency in gp91-*phox*. If the patient is a female, this in itself is an evidence 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 uncertainty as well. When both subunits of cytochrome b_{558} are detectable with the appropriate antibodies, a relative deficiency of NADPH

oxidase activity of the patients neutrophil membranes in the cell-free system will prove a defect in cytochrome b_{558}.

Diagnosis of carrier status for the autosomal forms is arduous, since the only biochemical finding is that the activity of neutrophils is lower than the activity revealed in normal controls. A definitive diagnostic tool is the detection of the mutation, once the mutation for a particular family is known.

The knowledge of the carrier status is useful for genetic counseling and prenatal diagnosis. The NBT test of the fetal neutrophils cannot be performed before 16 to 18 weeks of gestation. Instead, DNA analysis can be directly performed on amniotic cells or chronic villous samples right at the 12th or 20th week of pregnancy respectively. Obviously, this requires that the specific mutation is known in the family (Roos, 1996).

2.2 Mutations of the NADPH components

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 is an heterogeneous disorder. The transmission of CGD occurs either as an X-linked or an autosomal recessive character, depending on which subunit is altered.

In general, as described in a previous paragraph, patients with the cytochrome b_{558}-deficient form of CGD have a more severe clinical course than the ones with defects in cytosolic NADPH oxidase components, perhaps because of some residual electron transport also in absence of p47-*phox* and p67-*phox*. (1991; Forrest et al., 1988; Weening et al., 1985). However, it is in general difficult to predict the clinical course of the disease also because a residual activity of the NADPH oxidase in some patients is not correlated with the severity of the disease (Roos et al., 1992). This is probably due to the activity of auxiliary antimicrobial systems that play an important role in the microbial killing activity.

Defects in the other components of the oxidase like p40-*phox*, Rac1, Rac2 or GDP/GTP exchange proteins have not been identified so far. Mutations in these proteins may be incompatible with life, possibly because they are involved in other essential cellular functions.

Mutations in X-linked CGD

X-linked CGD is most commonly encountered, accounting for 50-60% of all CGD patients (Roos et al., 1996). The most frequent phenotype X91^{o} (50%) is the absence of the gp91-*phox* protein expression and the total absence of NADPH activity.

Occasionally other phenotypes occur, like the X91$^-$ (5-10%) or the X91$^+$ (< 5%) when subnormal or normal amounts respectively are detectable.

X linked CGD is a very heterogeneous disease, caused by a great variety of mutations represented by deletions, splice site mutations, missense mutations, nonsense mutations and insertions.

The X-CGD mutations database - An international study group was created in 1993 (Roos et al., 1996) to collect and classify the mutations involving the gp91-*phox* gene. The collection is composed by published and directly submitted data; the aim of the database is to facilitate the analysis of the gene and to understand the function-structure relationship in the protein.

To date, the database contains entries from 304 patients (261 families), for a total of 192 mutations (Roos et al., 1996).

Table V shows a comprehensive view of the mutations collected with the indication of the affected protein domains. Only two mutations have been found in the promoter region. The other mutations are equally distributed along the gene with a slightly lower frequency in the NADPH-binding domain and a slightly higher frequency in the N-terminal domain.

Mutations in the N-terminal domain are still under investigation for the effect they can have on the interaction with p22-*phox* and on the functionality of heme (Porter et al., 1996), but this part of the region definitely appears to be involved in membrane binding. Mutations in the FAD-binding domain have been studied for the interactions of this region with the cytosolic components of the oxidase (DeLeo et al., 1995; Leusen et al., 1994; Roos et al., 1996).

In the NADPH-binding domain, the mutations are clustered in one of the putative NADPH-binding sites, causing a reduced NADPH content or a diminished interaction with the cytosolic components (Roos et al., 1996; Segal et al., 1992).

In the small stretch of amino acids that compose the loop over NADPH binding cleft, the mutations impair again the binding of the cytosolic proteins (Leusen et al., 1994).

The analysis of this extended collection of data showed that single base-pair substitutions are the most frequent mutations, followed by deletions, insertions and partial duplications, in combination with small deletions and insertions. In frame deletions or insertions are very rare.

The most frequent mutation sites are Arg130 (seven families), Arg226 (seven families), Arg226 (seven families) and Arg157 (eight families), were the Arg codon is changed to a STOP codon. In the case of Ala84 the codon is changed from GCG to GCA in eight families. This silent mutation destroys the donor site of exon 3.

The most common mutated nucleotides are represented by the CpG dinucleotide (26% of the single nucleotide substitutions), changed to a CA, and by the insertion of an A in a row of six As, probably caused by a misparing at the DNA replication fork (Kunkel, 1990).

Table V. Mutations in the gp91-phox gene.
The numbers separated by slashes are: numbers of individual mutation types/number of families/number of patients with the mutation respectively. The percentages are for affected families. NA; not applicable. (from Roos D., 1996).

Type of mutation	Upstream	N-terminal domain	FAD-binding domain	NADPH-binding domain	Loop over NADPH-binding cleft	Total	% of total
Missense	2/2/3	22/26/29	11/15/16	11/15/16	1/1/1	47/59/68	22.6
Nonsense		18/46/51	17/15/18	9/9/9		34/70/78	26.8
Deletion inframe		1/3/3	1/2/3			2/5/6	2.0
Deletion frameshift		16/17/17	6/7/8	6/6/8	2/2/2	30/32/35	12.2
Large deletion						28/26/29	10.0
Insertion inframe			1/1/2	1/1/1		2/2/3	0.8
Insertion frameshift		12/19/22		4/4/4	1/1/2	17/24/28	9.2
Large insertion		1/1/1				1/1/1	0.4
Splice site inframe		13/21/27	3/4/8	1/1/1	1/1/2	18/27/38	10.3
Splice site frameshift		12/14/17	1/1/1			13/15/18	5.7
TOTAL	2/2/3	94/147/167	30/45/56	32/36/42	5/5/7		
Large deletion			28/26/29				
GRAND TOTAL						192/261/304	100

Deletions. - Deletions are the major cause of mutations in the X-CGD (32%) (Roos et al., 1996). They can vary from point mutations to big deletions involving the entire gp91-*phox* gene as well as neighboring genes, causing associated syndromes including Duchenne muscular dystrophy, retinitis pigmentosa and McLeods's syndrome. In most of the cases, deletions lead to the X91^{o} subtype of CGD. Partial deletions of the gene have been detected in patients with loss of different exons. As an interesting and particular example, two families with two sons each have been described, with the two mothers having different deletions in each X chromosome (mosaicism). As a consequence, the two respective sons showed different mutations. Incidentally, these two particular families underline the difficulties of prenatal diagnosis when a mosaicism is present in the mother.

In frame deletions represent 2% of the mutations and about 9% of the deletions detected so far (Roos et al., 1996). All these patients show a residual NADPH activity, thus suggesting that the in frame deletions affect the function of the protein, impairing the binding with FAD, NADPH or p22-*phox* or altering the structure of the protein itself, while they are still compatible with partial functionality (X91^{-} phenotype).

Many cases are characterized by single base pair deletions or deletions frameshift. In these cases, a premature termination of protein translation occurs and leads to the severe X91^{o} form.

Splice site mutations. - 56 of the patients so far described carry splice site mutations leading to a partial or complete absence of an exon in gp91-*phox* mRNA, representing the 16% of the X-CGD patients and leading to a severe form of the disease (Roos et al., 1996). Mutation in the 5' donor splice site are predominant. 76% of the patients have this kind of mutation, while 19% have mutations in the 3' splice site; only in one case was the mutation found to introduce a novel splice site (Roos et al., 1996).

The consequence of these mutations is the skipping of an exon during mRNA processing, usually leading to the formation of an unstable mRNA. Also the skipping of exons in the untranslated region (skipping of exon 13) have the same consequence (Royer-Pokora, 1996). In general, spice sites mutations lead to the X91^{o} phenotype, but there are cases with a residual protein activity (Roos et al., 1996). In a patient reported by Porter et al. (Porter et al., 1996) the skipping of exon 3 caused the absence of the mature protein but the presence of a gp91-*phox* precursor, leading to the conclusion that this region is important in the biosynthesis and maturation of gp91-*phox*.

Missense mutation. - Missense mutation are quite frequent in X-linked CGD, representing 22.6% of all the mutations (Roos et al., 1996). Although these mutations have no effect on mRNA stability, they affect the level and the function of cytochrome b_{558}, leading to the different phenotypes (X91^{o}, X91^{-} and X91^{+} CGD) (Roos et al., 1996). Different

polymorphisms have also been reported in the gp91-*phox* gene (Kuribayashi et al., 1996; Roos et al., 1996).

The $X91^+$ missense mutations are fundamental for understanding the structure-function relationship in gp91-*phox*. There are many examples of mutations of just one amino acid that can, in some cases, strongly affect the interactions of the protein with the other components of the complex and with the cofactors (Roos et al., 1996). In the NADPH binding domain for example, the mutation that leads to Pro415->His substitution has no effect on the stability of the protein but prevents NADPH binding; the Asp500->Gly substitution prevents NADPH activation without affecting the protein stability (Leusen, 1994). The latter mutation affects the domain involved in the binding of the cytosolic components of the complex, preventing their translocation. Mutations in the region between FAD and NADPH binding domains, and on the edge of the NADPH-binding region, affect p47-*phox* and p67-*phox* translocation, indicating that several regions are involved in this process. Another interesting mutation is the Arg54->Ser substitution, that affects the heme function of cytochrome b_{558} and consequently the electron transport from FAD to heme (Cross, 1995).

The mutations in the $X91^-$ CGD patients are in general located in the middle position of the protein (Roos et al., 1996). These mutations seem to decrease the stability of the protein, its association with the p22-*phox* subunit and its maturation, and the incorporation of the heme (Roos et al., 1996).

Two very interesting cases of the $X91^-$ phenotype have been described in which a small subset of neutrophils of the patients (5-15%) displayed normal oxidase activity (Newburger et al., 1994). In these patients two point mutations (A57->C and T55->C) have been identified in the gp91-*phox* promoter region, leading to a diminished expression of the gp91-*phox* mRNA. This effect was due to an alternative initiation site usage at nucleotide 190 of the normal cDNA sequence. Each mutation abolishes probably the association of the gp91-*phox* promoter region with a DNA-binding protein, but enhances the binding of another, larger DNA-binding protein.

Patients with missense mutations leading to complete absence of the protein ($X91^o$) have also been described (Roos et al., 1996). 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 histidine residues that might be involved in the heme binding.

Nonsense mutations - Nonsense mutations always induce the $X91^o$ phenotype, affecting also the mRNA level to various degrees. 78 patients carrying nonsense mutations have been described (Roos et al., 1996). Interestingly, most of them have C->T substitutions, changing the CGA codon for Arg into the TGA STOP codon.

These mutations also lead to a reduction of the mRNA levels from 0% to 50% without any correlation with the site of the mutations (Roos et al., 1996).

Insertions. - Insertions in the CYBB gene are less frequent than deletions, in accordance with findings in other genes (Copper and Krawczak, 1991). They represent 10% of the mutations (Roos et al., 1996). Usually they are single base insertion or insertions of few nucleotides. Only two in frame have insertions been detected so far.

In most cases, the mRNA stability is greatly decreased leading to a X91^{o} CGD subtype (Roos et al., 1996).

Mutation in autosomal CGD

Mutation of p22-phox. - Mutations that inactivate p22-*phox* lead to an autosomal recessive form of CGD. This type of CGD is rare, accounting for less than 10% of all CGD patients (Roos, 1994; Roos et al., 1996). The different phenotypes are designed as A22^{+} (<1%), A22^{o} (5-10%). Different defects have been described so far, similarly as for gp91-*phox* (Roos et al., 1996). Only in two patients from different families the same mutation was detected. Usually the level of mRNA is not affected, but the mutations lead to the formation of an unstable protein or of a protein that is unable to bind gp91-*phox*. Only in one case a large deletion was detected, removing almost the whole gene sequence and causing the absence of mRNA (Dinauer et al., 1990).

The only case of A22^{+} subtype is due to a Pro156 -> Glu substitution (Dinauer et al., 1991). This Pro is in a proline-rich region that is involved in the binding of the 47-*phox* through a SH3 domain. The mutation therefore impairs the translocation of the cytosolic proteins of the complex. In all the other cases, the complete absence of the protein leads to the A22^{o} phenotype. The mutations leading to this phenotype are mutations involving binding region for heme or for gp91-*phox*, or splice site deletions leading to exons skipping (Roos et al., 1996).

Mutations of the p47-phox. - Mutations in the p47-*phox* gene lead to the complete absence of the protein generating the A47° CGD phenotype. This type of CGD comprises about 30% of all CGD patients (Roos, 1994; Roos et al., 1996).

In contrast to the great heterogeneity of gp91-*phox* and p22-*phox*, only 4 different mutations have been found in 13 unrelated CGD patients with p47-*phox* deficiency (Roos et al., 1996). The genetic defects are represented by: i) a GT deletion in a tandem GTGT repeat, corresponding to the first four bases of exon 2; ii) 2 point mutations (A179->G and A425->G); iii) a deletion of G502 predicting a frame shift and introduction of a premature STOP codon.

Patients homozygous for the GT deletion present a frameshift and premature translation termination. There are also patients who are compound heterozygotes for the GT deletion in combination with the point mutations or the deletion described above. The mRNA is present in normal amount in these patients, while the p47-*phox* protein is always undetectable, suggesting that the produced protein is unstable.

On the other hand, a large number of polymorphisms have been detected in the p47-*phox* gene, most of which are silent, even if some of them lead to an aminoacid substitutions (Roos et al., 1996). This indicates that p47-*phox* is less dependent on a critical conformation for its function than the cytochrome b_{558} subunits.

Mutations of the p67-phox. - This CGD subtype is rare, accounting for less than 5% of all the CGD patients (Roos, 1994; Roos et al., 1996). Usually the mRNA level is normal, but there is no expression of the p67-*phox* protein. Therefore, most of the patients belong to the A67° CGD group (Kenney et al., 1993; Roos, 1994). Three silent polymorphisms have been found so far in the p67-*phox* gene (Kenney et al., 1993).

Introduction - part II

1. GENE THERAPY OF CGD

Long term prophylaxis with antibiotics, and in some cases subcutaneous γ-interferon, may reduce the frequency of serious infections in patients with CGD, but morbidity and early mortality remain significant (Mouy et al., 1991). Infusion of allogeneic leukocytes can also ameliorate the situation of some patients, although these treatments are always complicated by the development of allotypic antibodies.

Although in principle amenable to be treated by allogeneic bone marrow transplantation, this procedure has been successful in only a small number of patients, and the risks associated with this procedure outweigh potential benefits in the majority of cases (Hobbs et al., 1992). Morbidity and mortality relate to the high degree of immunosuppression required to achieve engraftment, and to graft versus host disease mediated by allogeneic donor T-lymphocytes. These problems could be circumvented by genetic modification of autologous cells with a functional copy of the defective gene. With this respect, CGD represents and ideal candidate disorder for gene therapy, since the disease has a recessive inheritance, and the disease phenotype is exclusively expressed in phagocytic cells.

Given the short half-life of mature phagocytes, the optimal target cell population for a gene therapy strategy for CGD is the pluripotent hematopoietic stem cell, defined by the capacity for extensive self-renewal and retention of multilineage differentiation potential.

Virus-mediated gene transfer of the correct gene for the defective NADPH oxidase component into this cell would, in principle, constitute definitive therapy. The genetically engineered stem cell can be returned to the bone marrow of patients, with subsequent production of corrected mature phagocytes. It can be expected that complete correction of respiratory burst activity in even as few as 10% of circulating phagocytes might decrease the susceptibility to infection in CGD patients, as inferred from studies of female carriers of X-linked CGD: some of these women, having less than 10% of normal cells, may display a normal phenotype (Roos et al., 1986). Consistent with this observation, also some CGD patients with even lower amount of residual phagocyte superoxide-generating activity have a mild clinical course, even if this is not the general rule (Roos et al., 1996) . These observations suggest that correction of only a small percentage of the cells in CGD patients will result in clinical improvement or cure.

On the other hand, however, also the relative level of respiratory burst oxidase activity within individual phagocytes may be an important determinant in the outcome of gene replacement therapy. Variant patients with X-CGD who have some residual respiratory burst activity can still develop severe infectious complications, and in vitro killing of *S. aureus* by

neutrophils from such patients has been shown to be markedly abnormal (Bu-Ghanim et al., 1995). These observations, together with experiments performed in a X-CGD mouse model (Bjorgvinsdottir et al., 1997), suggest that a partial correction of respiratory burst oxidase activity may not be sufficient to restore full microbicidal function to the phagocyte. Taking all these observations together, a gene therapy approach would ideally aim at the complete correction of oxidase production in at least 10% of the cells of the patient. Obviously, this is still, at the moment, a very ambitious task.

Alternative cell targets for gene transfer could also be autologous mature phagocytic cells, which can be transduced and reinfused into the patients. This approach aims at short term reconstitution of oxidase function, thus offering the advantage of avoiding the immune reaction which arises upon allogeneic cell infusions (Buescher and Gallin, 1982; Thrasher et al., 1995).

1.1 Pre-clinical models for gene therapy of CGD

Although the NADPH oxidase is a specialized enzyme of phagocytic cells, its expression has been reported in other cell types including fibroblasts (Meier et al., 1991), mesangial cells (Radeke et al., 1991), tonsillar B-lymphocytes (Maly et al., 1989) and EBV immortalized B-lymphocytes (Hancock et al., 1990; Leca et al., 1991; Maly et al., 1988; Maly et al., 1989; Pick et al., 1989). In addition, NADPH oxidase activity can be detected in transformed myeloid cells in vitro, which either maintain a partially differentiated myeloid phenotype or can be induced to differentiate by chemical treatments (Newburger et al., 1984; Zhen et al., 1993). Several of the above mentioned cellular systems can be used as in vitro models to test the efficacy of gene transfer procedures for gene therapy of CGD. In addition, the availability of methods for in vitro culture and differentiation of human hematopoietic progenitor cells, and the recent development of knock out mice with disruption of the gp91-*phox* gene (Pollock et al., 1995) and of the p47-*phox* gene (Jackson et al., 1995; Thrascher et al., unpublished), allow evaluation of the efficiency of functional reconstitution of oxidase activity in the primary target cells, in view of the final gene transfer application. The results obtained in these pre-clinical and experimental settings are summarized in Table VI and will be reviewed in the following sections.

Gene transfer into B-cell lines established from CGD patients

B-lymphocytes immortalized by EBV infection 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 phorbol myristate acetate, calcium ionophores and surface immunoglobulin cross-linking agents (Hancock et al., 1990; Leca et al., 1991; Maly et al., 1989). Although the magnitude of the response is roughly 1% of that of neutrophils, the

overall enzymatic process appears to be the same (Volkman et al., 1984). Furthermore, B-lymphoblasts from CGD patients are unable to support superoxide production (Orkin, 1989).

Due to the above mentioned properties, B-cell lines from CGD patients represent an invaluable in vitro model for the disease, both as a source of nucleic acids and proteins for the elucidation of the molecular basis of the defect, and, most notably, for the development of gene transfer protocols for gene therapy of this disease.

Several studies from different laboratories have demonstrated that protein expression and NADPH oxidase activity can be partially restored in EBV-transformed B-lymphocyte lines established from CGD patients after transduction or transfection with retroviruses or other expression vectors. Plasmid vectors expressing p47-*phox* (Chanock et al., 1992; Volpp and Lin, 1993) and p22-*phox* (Maly et al., 1993) have been shown to be able to reconstitute oxidase activity in B-cell lines with functional levels within the same range as in normal cells, with particular reference to those systems using EBV-based replicons able to be maintained as replicating episomes in these cells (Volpp and Lin, 1993). However, given the intrinsic difficulty of transfection of immortalized B-cells, these results could be obtained only after selection of transduced clones. For this reason, plasmid-based systems can be only taken to prove feasibility of genetic correction and have no practical usefulness, with the exception of representing an interesting tool to show the effects of specific mutations in the oxidase gene upon transfection of mutated cDNAs obtained from CGD patients (Chanock et al., 1996).

With the purpose of pre-clinical studies of gene therapy, several retroviral vectors have been developed and evaluated for expression of p47-*phox*, gp91-*phox* and p22-*phox* in B-cell lines derived from CGD patients. These vectors are based on the Moloney murine leukemia virus (MoMuLV (Li et al., 1994; Sekhsaria et al., 1993; Thrasher et al., 1992; Zentilin et al., 1996)), on the myeloproliferative sarcoma virus (MPSV (Porter et al., 1993; Porter et al., 1994)), and on the murine stem cell virus (MSCV (Ding et al., 1996)). The last two types of vectors have been chosen in order to confer a higher expression of the transferred gene in myeloid cells, with the perspective of gene transfer into hematopoietic precursors (see below). The overall survey of the data indicates that the levels of infection of B-cell lines by retroviral vectors is not satisfactory in terms of number of infected cells (probably reflecting a general refractoriness of cells of the lymphoid system to retrovirus-mediated gene transfer (Baker et al., 1992)). As a consequence, most of the studies have been performed by selection of transduced clones (Table VI). In these conditions, the levels of functional reconstitution of oxidase activity in NADPH-deficient cells are widely variable among the different studies, and approach in most cases the mean values measured in normal cells. Consistent with the observations made in myeloid cell lines - see below -, a general discrepancy has been detected between the levels of functional reconstitution obtained and the levels of protein expression driven by the transferred gene, which is generally low. For example, in the study described in this thesis, analysis of a number of clones obtained by transduction of X-CGD B-cells lines by a Moloney-based vector expressing gp91-*phox* showed functional restoration of superoxide production with average

Table IV. Classification of CGD

CGD subtype	Frequency (%)	Component affected	Heme spectrum	gp91-phox protein	p22-phox protein	p47-phox protein	p67-phox protein	Defect in cell-free system	Oxidase activity (% of normal
$X91^{0}$	50	gp91-phox	Absent	Absent	Trace	Normal	Normal	Membrane	0
$X91^{-}$	5-10	gp91-phox	Diminished	Diminished	Diminished	Normal	Normal	Membrane	10-30%
$X91^{+}$	<5	gp91-phox	Normal	Normal	Normal	Normal	Normal	Membrane	2-5%
$A22^{0}$	5-10	p22-phox	Absent	Absent	Absent	Normal	Normal	Membrane	2-3%
$A22^{+}$	<1	p22-phox	Normal	Normal	Normal	Normal	Normal	Membrane	0
$A47^{0}$	30	p47-phox	Normal	Normal	Normal	Absent	Diminished	Cytosol	0-2%
$A67^{0}$	5	p67-phox	Normal	Normal	Normal	Normal	Absent	Cytosol	0-2%

From Roos D. 1994.

levels similar to normal individuals, while protein expression was generally detectable at levels below 5% of normal (Zentilin et al., 1996). This notion again reinforces the suitability of CGD for a gene therapy approach.

A major obstacle to the presence of more than one open reading frame within a single retroviral vector backbone is promoter interference, which lowers either retroviral titer or transcription of one of the two genes (see below). A novel vector design to solve this problem is based on the use of bicistronic constructs, containing the downstream gene under the translational control of an internal ribosomal entry sequence. A similar type of vector has been developed by H.L. Malech and collaborators, containing the gp91-*phox* coding sequence and the mdr-1 gene, the latter to be used for selection of transduced cells (Sokolic et al., 1996). Populations of B-cell lines from an X-CGD patient transduced with this vector demonstrated very low initial levels of correction (0.1%-2.7% of normal EBV-B cell oxidase activity). However, selection for retrovirus-containing cells resulted in a population of cells which expressed oxidase values to a similar extent as normal controls. These type of studies are of general interest, since they indicate that a general strategy for gene therapy will be genetic correction even of a limited number of relevant cells, followed by amplification of the cured population by drug treatment. While the mdr-1 gene is a current possible candidate for this purpose (Baum et al., 1996), it is expected that in the next few years novel and more advantageous resistance gene-drug cognate combinations will be developed.

Another important avenue which is currently pursued for gene therapy of CGD, as well as for other genetic diseases of the hematopoietic system, is the use of vectors based on the adeno-associated virus (AAV). Among the proposed advantages of these vectors, is the presumed ability of these viruses of integrating in non-replicating cells (which appears to be very suitable for progenitor cells), their site-specific integration in the long arm of chromosome 19 (at least for the wild type virus), and the relative simplicity of their genome (Kotin, 1994; Samulski et al., 1991). An AAV vector, expressing the p47-*phox* coding region from the CMV promoter, has been indeed shown to be able to restore oxidase function in B-cell lines (Thrasher et al., 1995; Thrasher et al., 1995). However, several open questions about the usefulness of these vectors still need to be addressed, with special reference to their ability of infecting and integrating in hematopoietic progenitors (see later). Even for B-cell lines, the efficiency of infection in the absence of selection has been reported not to exceed 1% of the total cell population, with a consequent efficiency of reconstitution ranging from 1 to 2% of normal after 3 months of culture (Thrasher et al., 1995).

Taken altogether, the experiences which have accumulated in the recent years on gene transfer into CGD B-cell lines indicate that they still represent and excellent model for the first screening of developed vectors. However, quantitative data obtained in these cells have to be taken with caution, mostly because of the detected variability from clone to clone of the transfected cell, which partially reflects the natural variability of superoxide production among normal cell clones (Zentilin et al., 1996).

Gene transfer into established myeloid cell lines

The K562 cell line, derived from a patient with chronic myelogenous leukemia in blast crisis, exhibits multilineage markers, indicating that it is a highly undifferentiated, multipotent, hematopoietic precursor cell line (Lozzio and Lozzio, 1975). After appropriate stimulation, this cell line can undergo erythroid, megakaryocytic or granulocytic differentiation (Sutherland et al., 1986). K562 cells have been shown to constitutively express normal levels of p22-*phox* transcript, as well as of the Rac mRNA, similarly to a number of human tissues and cell lines (Parkos et al., 1988). These observations contrast with the specificity of expression of the other oxidase components, namely gp91-*phox*, p67-*phox* and p47-*phox*, which are limited primary to phagocytic lineages capable of generating superoxide. Given these properties, the K562 cell line represents an appropriate target to test reconstitution of the NADPH oxidase pathway upon transfer of the relevant genes. The complete oxidase system could be functionally reconstituted in transfected cells via cotransfection with episomal expression vectors (de Mendez and Leto, 1995).

A more specific cellular model of X-linked CGD was developed by Dinauer and collaborators (Zhen et al., 1993). The gp91-*phox* gene was disrupted by homologous recombination in a human myeloid leukemia cell line, PLB-985, which is bi-potential and can differentiate into either granulocytic or monocytic forms (Tucker et al., 1987). Targeted clones derived from the parental line do not display any respiratory-burst activity after granulocytic differentiation. Thus, this cell line offers the possibility to perform reconstitution experiments in a myeloid-cell environment, as well as to study the specific effects of gp91-*phox* mutants.

Functional correction in this cell line has been obtained by a retroviral vector expressing the gp91-*phox* cDNA (Kume and Dinauer, 1994). It is noteworthy that O_2^- formation in targeted clones containing the gp91-*phox* transgene varied in the range of 14% to 59% of the wild type, although the relative amount of recombinant gp91-*phox* protein, as evaluated by immunoblotting, was as little as 2% to 12% of wild type levels (Kume and Dinauer, 1994). These observations suggest that the cytochrome may not be the rate limiting component for respiratory-burst activity and that the expression of even modest amount of recombinant gp91-*phox* in X-CGD neutrophils may improve the function considerably. These findings are consistent with similar observations made in other cellular systems for in vitro reconstitution, most notably in EBV-transformed B-cells lines derived from the patients. Another interesting analogy between reconstituted B-cells and PLB-985 cells is the very variable levels of superoxide production which is scored among the different transduced clones (Zentilin et al., 1996). This variability is likely to be related to the different sites of integration of the retroviral vector, some of which may be less favorable for transcription. This conclusion is probably applicable to most gene therapy procedures utilizing retroviral vectors, with special reference to cell targets which need to undergo a differentiative process to express the corrected phenotype.

Very recently, better functional reconstitution of the superoxide function in the PLB-985 line has been achieved with a MSCV-based retroviral vector (Ding et al., 1996). With this vector, which confers myeloid-specific expression to the transgene, 57% to 119% of wild type levels of O_2^- production have been obtained.

Gene transfer into hematopoietic progenitors

Final correction of the NADPH oxidase defect for CGD will depend on successful gene transfer into hematopoietic stem cells. Blood progenitor cells from CGD patients with genetic defects in either the p67-*phox*, p47-*phox*, gp91-*phox* or p22-*phox* oxidase subunits have been successfully transduced using retroviral vectors (Table VI). Most of the reported experiences were performed by isolation of bone marrow or peripheral blood $CD34^+$ cells, transduction with the appropriate retroviral vector and evaluation of the infection efficiency and functional reconstitution upon differentiation of progenitor cells toward the myeloid lineage by addition of appropriate cytokine cocktails, either in liquid culture or in semisolid medium (Dick et al., 1991; Hughes et al., 1989; Metcalf et al., 1979). It should be stressed that this type of assay scores for the ability of an heterogeneous population of $CD34^+$ cells to differentiate in vitro, a property which is inherent to already committed hematopoietic progenitor cells but probably not to the primitive, pluripotent stem cell (Berardi et al., 1995). Given these considerations, the overall survey of the literature data indicates that the efficiency of infection of progenitor cells is generally extremely good, approaching 100% of transduced cells in some experiments (Ding et al., 1996; Zentilin et al., 1996). In contrast with the efficiency of infection, the evaluation of the efficiency of functional reconstitution of oxidase activity, measured both as the ability to generate superoxide in liquid cultures obtained upon myeloid differentiation of progenitor cells (Li et al., 1994; Porter et al., 1996; Sekhsaria et al., 1993; Weil et al., 1997) or by an application of the enzymatic NBT test to intact myeloid colonies in semisolid plates (Weil et al., 1997; Zentilin et al., 1996), shows poor restoration of the oxidase burst, rarely exceeding 1% of normal when Moloney-based vectors had been used (Ding et al., 1996; Li et al., 1994; Sekhsaria et al., 1993; Zentilin et al., 1996). Analysis of vector-driven RNA expression indicates that the primary limiting step in myeloid-differentiated cells is at the level of transcription of the transferred gene, since only very low levels of transcript could be detected from successfully transduced colonies (Zentilin et al., 1996).

From the comparative work on the different subunits of NADPH oxidase, it turns out that expression of the heterodimeric transmembrane subunits of flavocytochrome b_{558} (gp91-*phox* and p22-*phox*) in functional form is considerably less efficient than that of cytosol oxidase components (p47-*phox* and p67-*phox*) (Rotrosen et al., 1993; Weening et al., 1985; Weil et al., 1997). In fact, the assembly of a functionally active flavocytochrome b_{558} appears to be very demanding in molecular terms, since it requires coordinated expression of both subunits to achieve correct incorporation of heme and flavin moieties; disregulation in any of these events

Gene	Target cells	Vector	Additional genes in vector	Promoter of transgene	Selection of transduced clones	Functional reconstitution (% of normal)	Protein expression (% of normal)	Efficiency of infection (% of cells with trangene)	Reference
p22-*phox*	B-LCL	retrovirus (MPSV)	puro	vector LTR	yes	30%			Porter *et al.* 1994
p47-*phox*	B-LCL	EBV episome	hygro	CMV	yes	normal range			Volpp *et al.* 1994
p47-*phox*	B-LCL	retrovirus (MoMuLV)	neo	vector LTR	yes	30-36%	52%		Thrasher *et al.* 1992
p47-*phox*	B-LCL	AAV	-	CMV	no	1%			Thrasher *et al.* 1995a, b
p22-*phox*	B-LCL	EBV episome	hygro	SV40	yes	normal range	normal range		Maly *et al.* 1993
gp91-*phox*	B-LCL	retrovirus (MPSV)	puro	vector LTR	yes	10%	10%		Porter *et al.* 1993
p47-*phox*	B-LCL	retrovirus (MoMuLV)	neo	vector LTR	yes	no	<10%		Cobbs *et al.* 1992
p22-*phox*	CD34+	retrovirus (MoMuLV)	-	vector LTR	no	5%			Fei Li *et al.* 1994
gp91-*phox*			-		no	0.15-6.8%			
p22-*phox*	B-LCL		-		no	4.2-21.5%		10%	
gp91-*phox*			-		no	4.8-9.6%		10%	
p47-*phox*			-		no	7.8-9.1%		10%	
gp91-*phox*	X-CGD PLB985	retrovirus (MoMuLV)	-	PGK	no	14-59%	2-12%		Kume *et al.* 1994
p47-*phox*	CD34+	retrovirus (MoMuLV)	-	vector LTR	no	16.3%			Sekhsaria *et al.* 1993
	B-LCL				no	2.8-85%			
gp91-*phox*	X-CGD PLB985	retrovirus (MSCV)	neo	vector LTR	yes	57-119%	6-48%		Ding *et al.* 1996
	X-CGDmurine PBHP		neo		no	6-21%		100%	
gp91-*phox*	B-LCL	retrovirus (MoMuL,V; bicistronic)	mdr-1	vector LTR	yes	107-394%			Sokolic *et al.* 1996
	B-LCL		neo	vector LTR	yes	14-306%			
p47-*phox*	B-LCL	EBV episome	hygro	SV40	yes	5-10%			Chankock *et al.* 1992
gp91-*phox*	B-LCL	retrovirus (MoMuLV)	hygro	vector LTR	yes	30-100%			Zentilin *et al.* 1996
	CD34+				no	20%		60-100%	
gp91-*phox*	X-CGD PLB985	plasmid	hygro	EF-1a	yes	90%	5-20%		Zhen *et al.* 1993
gp91-*phox*	CD34+	retrovirus (MoMuLV)	-	vector LTR	no	very low		83-100%	Porter *et al.* 1996
p47-*phox*	monocytes	adenovirus	-	CMV	no				Thrasher *et al.* 1995c
p67-*phox*	CD34+	retrovirus (MFG)	-	vector LTR	no	25.3%	normal range		Wayne *et al.* 1997

Table VI. Gene tranfer experiments for the four CGD deficiencies in different target cell systems.
B-LCL: B-lymphoblastoid cell line; CD34+: CD34+ hematopoietic precursors; X-CGD PLB-985: gp91-phox-deficient PLB-985 cell line;
MoMuLV: Moloney murine leukemia virus; MPSV: myeloproliferative sarcoma virus; MSCV: murine stem cell virus

may account for the difficulties that have been encountered in recombinant expression of functional complex.

Gene transfer in primary monocytes

As a transitory gene therapy approach to autosomal CGD, A. Thrasher et al. (Thrasher et al., 1995) developed an adenovirus vector expressing p47-*phox*. This vector has been used to transduce the gene into monocytes from patients, and has been proven to be able to restore the NADPH activity in these cells. Contrary to retrovirus vectors, adenoviruses efficiently transduce non-dividing cells but they are unable to produce stable correction of progeny; therefore they appear appropriate for transitory gene delivery to differentiated cells, with the purpose of reinfusion of the engineered cells into specific body districts to solve clinically critical situations. Furthermore, transduction of primary monocytes by adenoviral vectors can be used as a system for the identification of defects within individual components of the NADPH oxidase and to confirm restoration of functional activity by gene transfer.

Due to its intrinsic limitations, however, this approach appears of limited use for curative therapy of CGD as well as of most other hematopoietic disorders.

Mouse model

An important achievement in the CGD field in the last few years has been the development of a mouse model for both the autosomal and the X-linked forms of the disease. Mice have been generated with a non functional allele for the gp91-*phox* subunit of the phagocyte oxidase using targeted homologous recombination in murine embryonic stem cells (Pollock et al., 1995). Respiratory burst activity is absent in neutrophils and macrophages obtained from affected hemizygous male mice. These mice also exhibit an increased susceptibility to infection with *S. aureus* and *A. fumigatus*, similar to human CGD, and have increased numbers of peritoneal exudate neutrophils during the chemical peritonitis induced by thioglycolate. Murine X-linked CGD appears to be a valid model for the human disease and will therefore represent a suitable in vivo system in which to investigate the pathogenesis of the clinical manifestations of this disorder and to refine treatment strategies. Experiments aiming at genetic correction of the disease in these mice indicate that the NADPH oxidase activity can be partially reconstituted using a MSCV retrovirus to transduce murine peripheral blood hematopoietic progenitors with the human gp91-*phox* cDNA upon in vitro differentiation (Ding et al., 1996). In the gp91-*phox* knock out model the correction via gene transfer of 50-80% of the neutrophils could protect the mouse against *A. fumigatus* challenge (Bjorgvinsdottir et al., 1997) Moreover, in mice translpanted with mixtures of wild type and X-CGD bone marrow cells, just 5% of normal neutrophils were required to correct the CGD phenotype. These results resemble the findings in female carriers with 5-10% of functional phagocytes.

A p47-*phox* knock out mouse has also been recently obtained, displaying the same phenotype as seen for human autosomal CGD, with development of infections resembling the pathologic features of human ones (Jackson et al., 1995). Recently, in this animal model, the infusion of progenitors transduced with a retroviral vector containing the p47-*phox* gene, demonstrated that a low percentage of corrected neutrophils (12%) could enhance the host defense against bacterial infections (Mardiney et al., 1997).

2. Retroviral vectors in gene therapy

Retroviral vectors provide a highly efficient method for gene transfer and stable incorporation of DNA into eukariotyc cells. Given their versatility, retroviruses are excellent tools for gene transfer in experimental systems and are potential vehicles for gene therapy in humans (Mulligan, 1993).

Although several different types of RNA and DNA viruses have been modified for the use as vectors for gene transfer, retroviral vectors are still the most frequently used in gene therapy clinical trials (Ali et al., 1994; Marshall, 1995). Indeed, Moloney-based retroviral vectors are being used in 71% of the protocols approved by the Recombinant DNA Advisory Committee in the USA. However, despite their frequent use, the choice of an appropriate gene therapy retroviral vector for a specific gene therapy application is still empirical. In addition, factors affecting virus titer and gene expression from retroviral vectors are still not completely understood.

Three viral genes are required in the virus formation: *gag*, coding for the capsid proteins, *pol*, coding for reverse transcriptase, protease and integrase, and *env*, coding for the viral surface glycoprotein (Coffin, 1996). Env determines the species-specificity of virus infection. Murine ecotropic *env* of Moloney murine leukemia virus (Mo-MuLV) allows infection of rodent cells, and amphotropic *env* (of MuLV 4070A) can target rodent, primate and some avian cells. Both the two *env* proteins recognize membrane receptors. The protein recognized by the ecotropic envelope is an amino acid transport protein (Albritton et al., 1989), while the receptor for the amphotropic virus is a sodium-dependent phosphate symport channel (Albritton et al., 1992; Kavanaugh et al., 1994).

The retroviral vector system can be divided into two components: the retroviral vector itself, which generally does not encode viral proteins, and the retrovirus packaging cell line, which provides the viral proteins necessary for vector transfer.

In a retroviral vector the viral genes have been almost totally removed, allowing the insertion of foreign genes. All the different classes of Mo-MuLV-based vectors contain the 5' and 3' LTRs, the packaging signal positioned just downstream from the 5' LTR and a portion of *gag* to increase titer of the vector (Adam and Miller, 1988; Armentano et al., 1987). The functions for packaging, reversetranscription and integration are provided in trans by the

packaging cell lines, that contain the *gag*, *env* and *pol* genes stably integrated in the genome (Miller et al., 1990). These cells are engineered in such a way, that the production of helper replication-competent viruses is avoided. This is achieved by minimising viral sequences in common with the vectors or putting the different proteins in different transcriptional units and replacing the LTRs with nonviral promoters and polyadenylation signals (Danos and Mulligan, 1988; Markowitz et al., 1988; Morgenstern and Land, 1990).

The effective utilization of retroviral vectors for gene transfer purposes is limited by several factors, including the titer of the virus, the level of the expression of the transgene, the complexity of the interaction between the provirus and the host genome (Barklis et al., 1986; Forestell et al., 1995; Hoeben et al., 1991; Jaenisch et al., 1981). Although the chromosomal site of integration is an important determinant of the expression of the integrated provirus, integration usually occurs in transcriptionally active sites of the genome (Feinstein et al., 1982; Rohdewohld et al., 1987; Vijaya et al., 1986). One of the major limitations of retroviral vectors is their ability to stably integrate only in replicating cells (Miller et al., 1990). Infact, the viral pre-integration complex is not able to be transported through the nuclear membrane and therefore it needs the nuclear membrane breakdown that occurs during mitosis to gain access to the cellular genome.

The expression of the transgene is instead correlated with the type of cells that are infected and with the particular vector used (Byun et al., 1996; Hantzopoulos et al., 1992; Malik et al., 1995). Considering all the above mentioned motives, many different types of retroviral vectors have been developed, to increase both the efficiency of infection and the efficiency of transcription of the therapeutic gene.

2.1 Strategies in retroviral vector design

Vectors with LTR-controlled expression

The physiological transcriptional control element of a retrovirus is contained in its long terminal repeat (LTR) at the 5' of the proviral genome, a sequence which is formed during the process of reverse transcription. In the LTR-based vectors, the gene of interest is expressed directly from this element, as either a spliced or an unspliced message. A selectable marker gene, if present, can be expressed from a differentially spliced message or from a polycistronic message (Dranoff et al., 1993; Krall et al., 1996).

Although the Mo-MuLV LTR is very active in most cell lines, the lack of gene expression from this promoter has been observed in several primary systems, including hematopoietic cells in vitro and animal models (Palmer et al., 1991; Richards and Huber, 1993; Williams et al., 1986). The Moloney LTR is completely inactive in embryonic stem and embryonic carcinoma cell lines, and the inactivity is accompanied by *de novo* methylation of the

proviral sequence (Challita et al., 1995; Jahner and Jaenisch, 1985). Moreover, the methylation has been detected in association with Mo-MuLV-LTR transcriptional inactivity in fibroblasts in vitro and in vivo (Hoeben et al., 1991; Palmer et al., 1991). Studies by Challita et al. (Challita and Kohn, 1994) demonstrated an LTR inactivity of 100% in the tertiary CFU-S (colony forming units) originated by hematopoietic progenitors, and this repression is strongly associated with methylation. In addition, the viral LTR have been shown to bind transcriptional repressors (Flanagan et al., 1989; Grez et al., 1990; Kempler et al., 1993). The transcriptional block is due to the presence of repressor elements located in the U3 region and in the primer binding site of the LTR.

Recently, different strategies have been developed to avoid promoter silencing of viral Mo-MuLV LTR, based on the use of LTR elements of other retroviruses, such as MESV (murine embryonic stem cell virus), MSCV(murine stem cell virus), MPSV(murine proliferative sarcoma virus), FMuLV (friend murine leukemia virus) or F-SFFV (friend spleen focus-forming virus), which are superior to Mo-MuLV for expression in hematopoietic cells (Baum et al., 1995; Grez et al., 1990; Hawley et al., 1994).

Internal promoter vectors

An alternative strategy for the expression of the therapeutic gene is the use of an internal heterologous promoter (Bowtell et al., 1988; Guild et al., 1988; Miller and Rosman, 1989; Morgenstern and Land, 1990). This strategy could potentially avoid problems of transcription in those cases in which the viral LTRs are inactivated or poorly functional. Vectors with internal promoters have been shown to function better in some cell lines than the LTR based vectors, yet in many cases have not resulted in high levels of expression (Byun et al., 1996; Hantzopoulos et al., 1992; Malik et al., 1995).

Different promoters within a retroviral vector can exert negative or positive effects on each other (Ghattas et al., 1991; Hatzoglou et al., 1991; Vile et al., 1994). Therefore, an important problem in this vector system is the possibility of promoter interference between the LTR and the internal promoter. This interference can increase the transcription or completely inactivate the expression from the internal promoter. Interestingly, also the site of integration of the provirus can influence the selection of the active promoter (Hatzoglou et al., 1991). Furthermore, some experiments demonstrated that the sequence of the gene cloned in the retroviral vector exerts some influence in the transcriptional activity of the two promoters as well (Bowtell et al., 1988; Kume and Dinauer, 1994). Also the loss of specificity of expression from the internal promoter, when used in a retroviral vector, probably reflects these complex interactions between promoters (Vile et al., 1994; Vile et al., 1995). Another strikingly important problem is that the presence of the internal promoter usually has a strong influence on the virus titer, especially when in the packaging system transcription is not avoided from the internal promoter (Byun et al., 1996).

To overcome the problem of transcriptional interference, self-inactivating vectors (SIN) have been developed, in which the LTR enhancer-promoter gets deleted upon infection (Soriano et al., 1991). These constructs allow transcription to start from the internal promoter without the interference of the LTR. Unfortunately, most authors have reported very low titers with these vectors, which still prevent their general application. More recently, SIN vectors have been generated based on the Cre-lox system that exploits the ability of the P1 phage site-specific recombinase Cre to excise any sequences positioned between two loxP target sequences from the mammalian genome (Russ et al., 1996).

Bicistronic vectors

A considerable problem using an internal promoter for the expression of a selectable marker is that, the selection for the resistance protein can enrich for retrovirus transduced cells, but it does not ensure expression of the therapeutic gene. Infact, it is well known that the selection for a promoter positioned 3' to another promoter can suppress transcription of the nonselectable gene from the upstream promoter (Emerman and Temin, 1986; Emerman and Temin, 1986). Therefore, a strategy aimed to increase the expression of the therapeutic gene is to link the two genes via an internal ribosome entry site (IRES) element (Chen et al., 1993; Morgan et al., 1992). The presence of a single transcription unit dependent on a single promoter avoids any promoter interference. IRES elements are 500-600 bp sequences that are characteristic of the 5' untranslated regions of picornaviruses, including poliovirus and encephalomyocarditis virus (EMCV) (Davies and Kaufman, 1992; Sonenberg, 1990). IRES elements can pick up to three genes in a retrovirus vector without the use of internal promoters. The use of an IRES instead of an internal promoter ensures retention of translation of the therapeutic protein during selection for the resistance protein (Sokolic et al., 1996). .

Other strategies in the retroviral vector design

The reverse orientation vector is a construct where the gene of interest is inserted in reverse orientation and it is expressed using its own promoter and polyadenylation signals (Miller et al., 1988). However, the use of this kind of vectors is restricted to specific applications.

Another strategy consists of creating a chimeric protein resulting from fusing a sequence from the open reading frame of a resistance gene and a therapeutic gene (Germann et al., 1990). Depending on the gene products fused, this strategy may be successful, or can interfere with the function of either the therapeutic protein or the resistance protein, because the conformation of either may be modified in the chimera.

The double copy vector is a construct in which the gene of interest and a promoter are inserted in the U3 region of the 3' viral LTR (Hantzopoulos et al., 1992). Due to the particular

mechanism of reversetranscription of retroviruses, the insertion is duplicated also in the 5' LTR, generating two copies of the therapeutic gene and associated promoter (Coffin, 1996). The short length of the insertions generally used does not affect the retroviral transcription from the LTR during the production of the retrovirus. Instead the duplication of the gene ensure high expression of the gene itself (Wolfe et al., 1995).

Retroviruses using alternative receptors and pseudotyped vectors

The poor efficiency of infection of amphotropic retroviruses for some tissues, such as those of the hematopoietic system, may be a consequence of the low expression of the amphotropic receptor in these cells (Orlic et al., 1996; von Kalle et al., 1994).

As an alternative to amphotropic retrovirus-mediated gene transfer, transduction of these cells can be improved by the use of different retrovirus vectors. The gibbon ape leukemia virus (Miller et al., 1991) uses as a receptor a different phosphate channel protein (O'Hara et al., 1990), which is highly homologous to the phosphate channel protein that serves as the amphotropic retrovirus receptor (Kavanaugh et al., 1994). Gibbon ape leukemia virus has been adapted as gene transfer vector and has been used to transduce human hematopoietic cells at levels that are 2 to 3 fold higher than those obtained with amphotropic retrovirus vectors (Bauer et al., 1995; Bunnell et al., 1995; Miller and Miller, 1994; von Kalle et al., 1994).

An alternative strategy to increase the efficiency of infection of the retroviral particle is to pseudotype the virus by the use of envelope glyproteins derived from the vesicular stomatitis virus (VSV). The VSV envelope glycoprotein (VSV-G) mediate transduction a wide variety of tissue culture cells (Burns et al., 1993; Hopkins, 1993; Ory et al., 1996; Yee et al., 1994), and, since the receptors for VSV-G are likely to be membrane lipids (Schlegel et al., 1983), their numbers on the target cells should not be limiting.While this system has several advantages in terms of the production of high titer infectious preparations, it has been hampered by the fact that the VSV-G protein is highly toxic for most cultured cells, and therephore also for the packaging cells. Recently, new packaging cell lines have been developed, in which the VSV-G protein is produced under the control of an inducible promoter (Ory et al., 1996)

Finally, modifications of the viral envelope are also desirable for in vivo utilization of retroviral vectors. In fact, these vectors appears to be rapidly inactivated by human serum, most likely through the action of complement. Therefore, attempts are currently made for the construction of viruses that can be serum-resistant, and that can be utilized for in vivo purposes (Cosset et al., 1995; Takeuchi et al., 1996).

Targeted retroviral vectors

The development of retroviral vectors with a defined target cell specificity can be achieved at two different stages (Schnierle and Groner, 1996). A targeted expression of the

transgene can be obtained introducing in the promoter transcriptional regulatory elements, that are recognized by specific transcription factors in the target tissue (Valerio et al., 1989; Vile et al., 1994; Vile et al., 1995). The regulatory elements can be introduced either in the viral LTR or in the internal promoter This gives the potential of regulating the expression of the gene inserted within the provirus (see above). The second possibility of targeting a retroviral vector to a specific tissue can be achieved at the infection level. Retroviral vectors packaged by cells expressing a modified envelope protein are being developed for more specific transduction. This kind of retroviral vectors should limit the gene transfer to a desired cell type, allowing their utilization for *in vivo* transduction. Infact, targeting to specific cell types is so poor with existing retroviral vectors, that usually the procedures involve *ex vivo* transduction. By modifying the envelope spike glycoprotein of murine leukemia viruses, it may be possible to increase both the selectivity and the efficiency of gene transfer targeting the virus to a specific cell. The final objective of these vectors is the in vivo utilization.

Some authors reported the generation of retroviral vectors incorporating a chimeric envelope glycoprotein in which a single chain anti-apten antibody fragment or a receptor for a membrane protein were fused to the envelope protein (Ager et al., 1996; Cosset et al., 1995; Kasahara et al., 1994; Nilson et al., 1996; Schnierle and Groner, 1996). Unfortunately, very low vector titers are obtained with this procedure, and the co-incorporation of unmodified Mo-MLV envelopes is indispensable for a detectable vector infectivity. These results suggest that unknown functions of the Moloney glycoprotein might be affected by these kinds of modifications.

3. GENE THERAPY FOR HEMATOPOIETIC CELLS

The ideal target cell for gene therapy of hematopoietic disorders should be able to renew itself and differentiate into progeny cells after transplantation to generate a sizable self-perpetuating cell mass, that contains the transferred gene for the entire lifespan of the patient. Bone marrow transplantation experiments in humans and animals show that the repopulating bone marrow stem cell fulfills all these criteria (Huang and Terstappen, 1994; Karlsson, 1991). Many attempts to characterize the most primitive hematopoietic cell were able to identify it as a cell expressing the CD34 membrane antigen, but not the CD38 membrane antigen (Berardi et al., 1995; Rusten et al., 1994; Terstappen et al., 1991).

Most studies, over the past 10 years, have used highly infectious retrovirus vectors to introduce genes into hematopoietic stem cells (Barranger, 1996; Karlsson, 1991; Kohn, 1995). Methods for retrovirus-mediated gene transfer were first developed using mouse bone marrow because of the availability of repopulation assays for murine pluripotent stem cells. These studies showed that retroviruses could efficiently transduce pluripotent stem cells, that

completely reconstituted the entire hematopoietic system of the mouse (Bodine et al., 1996; Correll et al., 1992).

However, both these studies and the studies on the primate hematopoietic progenitors (van Beusechem and Valerio, 1996), revealed a certain number of problems and left questions still open, mainly concerning the efficiency of gene transfer ex vivo in the truly undifferentiated progenitor cells. Inefficient infection of hematopoietic stem cells (HSCs) is due to several problems: HSCs are rare, being estimated less than 1% of $CD34^+$ bone marrow cells (Szilvassy et al., 1990), they divide infrequently (Larochelle et al., 1996). A loss of expression of the transferred DNA with time occurs, that may be attributed to the infection of more mature progenitors, which possess higher cycling rates and a progeny of limited lifespan. In addition, expression of introduced sequences in primary hematopoietic cells has been problematic (Apperley et al., 1991). In some cases retroviral vectors have even consistently failed to direct expression of the introduced genes in vivo, despite substantial expression in cell lines in vitro (Szilvassy and Cory, 1994; Williams et al., 1986).

The ability of the retroviruses to infect HSC is dependent on a number of factors including duration of cocultivation, the packaging cell line and the cycling status of the cell population (Miller et al., 1990; Van Beusechem et al., 1993; Xu et al., 1994).

3.1 Problems regarding infection of the hematopoietic progenitor

An important fact causing the poor transduction efficiency is that retroviral integration is inefficient or absent in non-replicating target cells (Miller et al., 1990). Pluripotent HSCs in bone marrow tend to be physiologically in G_0 or G_1 (Knaan-Shanzer et al., 1996) and, therefore, they are difficult targets for retroviral vectors. Control of pluripotent HSC renewal in normal resting cells is poorly understood at present, but it is assumed to be controlled by the range and concentrations of hematopoietic growth factors in extracellular matrix surrounding bone marrow stromal cells (Metcalf, 1993; Metcalf and Nicola, 1991; Whetton and Dexter, 1993). Although the conditions for transduction of pluripotent HSCs are still not standardized, many attempts have been done to understand the effect of cytokines used during the infection protocol, and the conditions that can preserve the renewal capacity of the stem cell (Bodine et al., 1989; Luskey et al., 1992; McKenna et al., 1995; Szilvassy and Cory, 1994; van Beusechem et al., 1995). Infact, the exposure of bone marrow or peripheral blood stem cells to the currently used culture systems and cytokine cocktails may create an engraftment defect in the target HSCs and may lead to a loss of repopulating capacity of bone marrow cells during the infection (Dumenil et al., 1989; Yonemura et al., 1996). This can be assigned to the culture conditions which induce differentiation in the progenitor cells. Several ameliorants are expected in this field in the next ten years, including the utilization of stromal cell support, the negative

selection of already committed progenitors, the positive selection of engineered stem cells, and the use of alternative viral vectors.

Utilization of stromal cells. Recent studies using stromal cell support have demonstrated the enhanced efficiency of retroviral transduction of primitive, long lived progenitors and the maintenance of long term repopulating cells in this kind of culture (Nolta et al., 1995; Wells et al., 1995; Xu et al., 1995). The importance of stroma in the activation of cycling of the hematopoietic progenitors is evidenced by the enhanced transduction efficiency of the bone marrow progenitors. The precursors obtained instead from mobilized peripheral blood cell are easily transduced, also in absence of stroma. Infact, the cells obtained from peripheral blood seem to have a higher fraction of proliferative committed progenitors (Sekhsaria et al., 1993).

Interestingly, recently Wineman and collaborators (Wineman et al., 1996) showed that the stroma microenvironment is not homogeneous, being composed by different kind of cells among which just few interact selectively with the stem cells. The adherence of hematopoietic stem cells to the extracellular matrix of the bone marrow environment is fundamental for their maintenance and it is mediated by several interactions (Wineman et al., 1996; Yoder and Williams, 1995). One interaction involves fibronectin, a protein that is very abundant in bone marrow microenvironment. Fibronectin (Moritz et al., 1994) and integrins (Williams et al., 1991) can also stimulate activation via tyrosine phosphorilation and cytoplasmatic alkalization in response to attachment of various cell type to extracellular matrix molecules. Interestingly, retroviruses are able to bind to fibronectin and this increases gene transfer of long term hematopoietic progenitors (Moritz et al., 1996). A co-localization effect is probably responsible for the increased infection (Hanenberg et al., 1996). Recently, it has been demonstrated that upon cytokine induction of $CD34^+$ cells the fibronectin receptors are activated (Levesque et al., 1996). These findings show that activation of fibronectin receptors is correlated with the replicative state of the cells giving an important elucidation in the understanding of hematopoietic stem cells activation.

Long term culture conditions. One method for the culture of hematopoietic precursors that can allow the recruiting of replicating cells for the transduction is the long-term bone marrow culture (LTBMC) (Emerson, 1996). This procedure, that can be performed both in absence and in presence of stroma, allows the infection of rapidly dividing progenitors (Bienzle et al., 1994; Wells et al., 1995). This method can take advantage of transferring potentially therapeutic genes to HSCs, that are highly proliferating and responsible for the reconstitution event in the non ablated recipient. The efficiency of transduction obtained in this system can be assigned to the long time (5 weeks) in which the cells are kept in culture. Infact, in a short term culture also the committed progenitors are present, while they are eliminated in a long term culture upon differentiation. The committed progenitors and the already differentiated cells have a negative influence on the uncommitted precursors, inhibiting their replication (Fraser et al., 1990;

Pawliuk et al., 1996). The efficiency of this method has been already demonstrated in canine models (Bienzle et al., 1994) and it is currently being evaluated in a marking study in myeloma patients (Stewart, 1995).

Selection of the transduced cells. The in vitro selection after transduction with a retroviral vector carrying a resistance gene is a possibility to increase the number of transduced cells which shall be transplanted. For this purpose retroviral vectors containing a number of resistance genes that allow the enrichment for the transduced cells have been developed. The most commonly used resistance genes are enzymes that confers resistance to toxic compounds such as neomycin or hygromycin (Palmer et al., 1987). However, selection with these markers have disadvantages like toxicity and difficulties in quantitative expression levels (Valera et al., 1994). On the other hand, some resistance genes allow the selection in vivo of the cells that have been transduced, giving them a growing advantage. Vectors containing a mutated DHFR gene can confer methotrexate resistance to the transduced cells. Also the mdr-1 (multidrug resistance) gene can be used as a resistance marker (Baum et al., 1996; Li et al., 1994; Sorrentino et al., 1995). It codes for a natural human protein and can be used both for the in vitro selection during expansion of engineered cells and for the in vivo selection to increase the number of engineered cells relative to the non-engineered ones after infusion into the patients. Selective agents that could be administered to patients include vincristine, taxol and daunorubicin. Taxol has been already successfully used for this aim in a mouse model (Sorrentino et al., 1992).

Genes encoding cell surface antigens can also be used as selectable markers (Mavilio et al., 1994; Pawliuk et al., 1994). The advantage of this kind of markers is the efficient and nontoxic selection by FACS (fluorescence activated cell sorter), that allows also a quantitative analysis of the transcription and the possibility to follow the fate of the transduced cells, once they have been reinfused in the patients.

Alternative viral vectors. The low level of amphotropic retrovirus-mediated gene transfer into human hematopoietic stem cells has been a major impediment to gene therapy for hematopoietic diseases. Recent studies in mice have shown that in the most primitive hematopoietic cells ($CD34^{+}CD38^{-}$) the amphotropic receptor is very poorly expressed and the level of amphotropic receptor mRNA is very low compared to the mRNA for the ecotropic receptor. Accordingly, the efficiency of transduction of murine cells with an ecotropic virus is much higher than the one obtained with an amphotropic virus. In a related study in human populations of HSCs they showed that the amount of human amphoR mRNA was very low (Orlic et al., 1996).

There are several potential solutions to the problem of low level expression of amphotropic receptor mRNA. One possibility is to select a subpopulation of mouse HSCs expressing higher levels of receptor mRNA, which are more efficiently transduced (Orlic et al., 1996). Alternatively, the number of amphotropic retrovirus receptors on HSCs might be increased by inducing their mRNA expression. Kavanaugh et al. (Kavanaugh et al., 1994)

showed that mouse mRNA expression can be increased by culturing the cells in phosphate free medium. Crooks et al. (Crooks and Kohn, 1993) showed that binding of amphotropic viruses to human $CD34^+$ bone marrow cells can be increased by culturing the cells with the cytokines SCF, IL-3 and IL-6. Orlic et al. (Orlic et al., 1996) demonstrated that the receptor mRNA level can be increased by culture in growth factors. A physiological increase in the receptor levels can be observed in more differentiated $CD34^+CD38^+$ cells, and this could be another reason for the high efficiency in transduction of committed progenitor cells, but not of long-term repopulating cells.

As an alternative to amphotropic retrovirus-mediated gene transfer, transduction of HSCs may be more efficient with different retroviral systems that recognize different cell surface receptors. In this respect, the retroviral vectors containing the gibbon ape leukemia virus envelope protein or the vesicular stomatitis virus envelope protein have been already described above.

Further to the receptor abundance problem, still the pluripotent HSC is likely to be a non-cycling cell, and thus to be refractory to infection by animal retroviruses. Thus, novel strategies for gene transfer in these cells are highly desirable. A particularly attractive gene delivery system is the one based on the adeno-associated virus (AAV). These viruses have many attributes including a broad tissue tropism, the ability to integrate stably into the host genome and the potential for efficient infection of non dividing cells, that render them attractive for gene therapy of pluripotent HSCs (Berns and Linden, 1995). However, the wider application of rAAV gene transfer is at present limited by inefficient methods for the generation of transducing recombinant viral particles, as well as by the still unsatisfactory understanding of the molecular biology of the viral life cycle.

Other suitable vectors for the transduction of non cycling cells are lentiviral vectors, most notably HIV-1 (Naldini et al., 1996; Poeschla et al., 1996). The advantage of these vectors in the infection of resting cells is due to the ability of their pre-integration complexes to cross the nuclear envelope through the nuclear targeting properties of the proteins p17 Gag and Vpr (Gallay et al., 1995; Heinzinger et al., 1994). However, both complete lentiviral vectors and pseudotyped-lentiviral vectors are still limited in their efficiency of infection, which is at present unsatisfactory for clinical applications.

3.2 Functional characterization of HSCs

A major problem that compromises the field in its approaches to transduction of human self-renewing pluripotent HSCs is that there are neither phenotypic markers available for these cells nor in vitro assays. Stem cells can only be assayed by their ability to proliferate and differentiate into all blood lineages after transplantation into another recipient (Szilvassy et al., 1990). In vitro cultures are not capable of measuring human stem cell activity. Most of the

currently employed gene transfer methods used in the human clinical trials have been optimized using the in vitro clonogenic assay and LTC-IC assays (Hughes et al., 1989; Metcalf, 1977; Verfaille and Miller, 1995).

In the in vitro methylcellulose assay the progenitor cells (CFU-C; colony forming cells CFCs), are scored for their ability to give rise to colonies representing different hematopoietic lineages. The different colonies are then analyzed for the presence of the retrovirus and the expression of the transduced gene. In the long term culture initiating cells (LTC-IC) the cells are assayed as colonies grown from progenitors surviving in long term marrow culture 5 weeks after transduction. However, stem cells do not share the same biological properties as progenitors cells, e.g., proliferative and self renewal capacity, response to growth factors, cell surface markers. Thus, these methods may have little relevance for stem cell gene transfer. Furthermore, in human and non-human primates reconstitution studies are either ethically prohibited or too costly to be widely applied. A reasonable experimental model can be instead, the assay for human SCID-repopulating cells, namely the reconstitution of human hematopoiesis in the context of immunodeficient mice (SCID or NOD-SCID mice) (Larochelle et al., 1996; Larochelle et al., 1995; Nolta et al., 1996; Salomon et al., 1990; Vormoor et al., 1994). High levels of committed and multilineage myeloerythroid progenitors, as well as mature human myeloid, erythroid and B-cells develop in the murine bone marrow and are maintained for at least 4 months. Intravenous injection of human bone or cord blood into these mice result in the repopulation of the animals with these human cells. These primitive cells have been denominated SCID repopulating cells (SRCs).

The SRC that initiates the graft is very primitive and kinetic experiments suggest these cells are earlier in ontogeny than colony-forming progenitors (Plavec et al., 1996). Using gene marking, Larochelle et al. (Larochelle et al., 1996) characterized the repopulating ability of LTC-IC, CFC and SRC and demonstrated that LTC-IC and CFC are not able to repopulate SCID mice. Moreover, SRC are found in the $CD34^{+}$ $CD38^{-}$ enriched population. On the other hand, SRC are poorly transduced compared with the other two cell types, demonstrating a low level of gene marking in the animals. Also in another animal model, the human-sheep model, a subset of $CD34^{+}CD38^{-}$ injected in a preimmune fetal sheep demonstrated, that indeed this fraction has a high capacity for long-term multilineage hematopoietic engraftment (Civin et al., 1996). Although correlation to human or non-human primate reconstitution remains to be demonstrated, these transplantation systems provides a powerful tool to characterize primitive human cells and to create animal models of human hematopoietic diseases (Dick et al., 1991; Lapidot et al., 1994).

3.3 Outcome of gene therapy and marking trials

Although many studies have assessed the feasibility of infection of progenitors and subsequent engraftment in mice, the same methods were much less efficient for bigger animals or humans stem cells, where the level of genetically marked cells after reconstitution were generally lower than 1% (Bodine et al., 1993; Cornetta et al., 1996; Kohn, 1995; Van Beusechem et al., 1993; van Beusechem et al., 1992; van Beusechem and Valerio, 1996). The early experience in human clinical trials and in gene marking studies (Brenner, 1996; Dumbar et al., 1995) also suggested that gene transfer into normal human stem cells is inefficient. Therefore, significant species differences exist in the efficiency of retrovirus infection, pointing out to the importance of extensive preclinical evaluation of vectors and conditions specific for human stem cell gene transfer.

The reasons of the discrepancy between reconstitution in mice and in large animals are still not clear. In large animals the number of long term in vivo repopulating stem cells is at least one and in most instance two orders of magnitude lower (Abkowitz et al., 1995; van Beusechem and Valerio, 1996). Additionally, the low efficiency of repopulation obtained is probably due the particular development of hematopoiesis in vivo. There is evidence that hematopoietic recovery after bone marrow transplantation initially involves numerous progenitor cells, each contributing to clonal progeny of the blood cellular elements. Hematopoiesis has been characterized after autologous transplantation of marrow cells in a recipient (humans, mice, dogs, and cats) using gene marking or after autologous transplantation. The analysis showed fluctuations in clonal population followed by the emergence of a stable clone. Infact, with time the cells of some of these clones undergo terminal differentiation and death (clonal extinction), and hematopoiesis becomes oligoclonal. The initial disequilibrium extends for 1-4 years in large animals (Abkowitz et al., 1996; Abkowitz et al., 1995; Van Beusechem et al., 1993), which is in contrast with the short disequilibrium interval (2-6 months) seen in comparable murine studies (Nash et al., 1988; Snodgrass and Keller, 1987; Terstappen et al., 1991).

A contradictory hypothesis instead suggests that, if clonal succession of stem cells is the explanation for the lower transduction efficiency observed in primates, one would expect to see peaks in the percentage of provirus-carrying cells, reaching values comparable to what is found in mice. This has not been observed. If clonal extinction of transduced HSCs were to occur, this would lead to the complete disappearance of provirus carrying cells. Thus, the low percentage of transduced cells is an indicator of poor transduction efficiency.

There are still contradictory results concerning the necessity of bone marrow ablation in the recipient patient. Ablation was considered a prerequisite to obtain maximum engraftment for a long time (Barquinero et al., 1995; Hoogerbrugge et al., 1996; Lenarsky and Parkman, 1990; Lenarsky et al., 1990; Spain and Mulligan, 1992). However, some experiments in mice and dogs demonstrated that bone marrow ablation is not required for a successful engraftment

of the transduced cells (Bienzle et al., 1994; Schiffmann et al., 1995; Stewart, 1993). These experiment utilized long-term cultures for the infection of the hematopoietic precursors. The authors suggested that the activated cycling progenitors obtained from the long term culture can compete with endogenous cells, because they continue normal proliferation in vivo.

Bone marrow ablation has significant associated morbidity and mortality (Armitage, 1994). Therefore, it would be greatly advantageous to avoid this preparatory regiment before transplantation. Aggressive bone marrow ablation using irradiation or chemotherapy before transplantation may be problematic in severely ill patients such as it is in Gaucher disease or in patients who have an immune disorder such as CGD. Therefore, transfer of the corrected gene without bone marrow ablation is an attractive treatment alternative. Because allogenic bone marrow transplantation has been successful in many diseases involving the hematopoietic system and other genetic metabolic diseases, a permanent cure would be expected, if a sufficient proportion of stem cell population is transduced with a vector containing the normal gene.

As an alternative to repeated bone marrow harvest, peripheral blood hematopoietic progenitors can substitute for bone marrow in autologous transplants leading to permanent reconstitution of hematopoietic function indicating that earliest stem cells are represented in this compartment (Kessinger et al., 1988; Moritz et al., 1993). Apheresis collection of peripheral blood hematopoietic progenitors is atraumatic and can be repeated frequently over a long time. Repeated collection, transduction and reinfusion of corrected progenitors might be of benefit. Of relevance to current studies, peripheral blood hematopoietic progenitors appear to be a receptive target for retrovirus-mediated gene transfer, suggesting a potential role of these cells as a bone marrow substitute for somatic gene therapy (Bregni et al., 1992; Sekhsaria et al., 1993).

The incomplete understanding of the hematopoietic stem cell biology contributes to the obstacles in the path. Thus, further work at the molecular level is needed to uncover aspects of hematopoietic stem cells life that can be exploited to increase their benefits in the clinical application.

Materials and Methods

1. Materials and cell lines

RPMI 1640 and Iscove's modified Dulbecco's medium (IMDM) were obtained from Gibco BRL Life Technologies LTD (Paisley, Scotland); fetal calf serum was obtained from Hyclone Laboratories (Logan UT); rhIL3, rhIL6, rhGM-CSF, rhSCF were obtained from Genzyme (Cambridge, MA); labelled (^{32}P, ^{35}S) deoxynucleotides were obtained from Amersham International plc (Amersham, UK); Nytran membranes were obtained from Schleicher & Schuell (Keen, NH); endoglycosidase F was obtained from Boehringer Mannheim (Mannheim, Germany); hygromycin B, polybrene, methylcellulose, 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 work were synthesized by the ICGEB Oligonucleotide Synthesis Service on an Applied Biosystem 380B synthesizer.
The PA317 murine amphotropic packaging 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 (Mann et al., 1983) was kindly provided by Dr. Maria Pia Grossi, University of Ferrara, Italy. PLB-985 and PLB-985 X-CGD was a kind gift of M. Dinauer.
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 antibody MoAb 449, directed against gp91-*phox*(Verhoeven et al., 1989), was a kind gift of Dr. Verhoeven, Academic Medical Center, University of Amsterdam, The Netherlands.
Plasmids pCR^{TM}II vector and pCEP4 are from Invitrogen (San Diego, California). The plasmid pBLCAT 3 was a kind gift of Dr. Laurence Banks, ICGEB Trieste.

2. Establishment of B-lymphoblastoid cell lines

Separation of PBMCs

10 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 Ficoll Hypaque (Sigma, S.louis, MI, USA) and centrifuged for 20 min. at 1000 g. The peripheral blood mononuclear cells (PBMCs) ring was recovered and washed three times with RPMI 1640

Establishment of lymphoblastoid cultures

The EBV-infected marmoset cell line B95-8 was used as a source of EBV. Cells were grown until a concentration of 10^6/ml, and the EBV-containing supernatant was harvested by centrifugation at 250 g to remove cells and debris. The supernatant was then passed twice through a 0.45 mM membrane filter (Millex, Millipore). This virus preparation can be kept at 4°C for several months. Just before use the supernatant was diluted 1:1 with fresh medium containing 50 µg/ml gentamicin, 10% fetal calf serum and 2 mM L-glutamine.
Total PBMCs were resuspended in the supernatant of the B95-8 cells line diluted 1:1 with fresh medium, at a cell concentration of $2x10^6$/ml. Two ml of culture was established. 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. (Neitzel, 1986).

3. CHARACTERIZATION OF THE GENETIC DEFECT

Superoxide production assay.

The luminol assay for testing superoxide production was performed according to Porter et al. (Porter et al., 1992). 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, 1mM $CaCl_2$, 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 counter. Luminol was added just before the first measurement (time 0) at a final concentration of 9 mM. 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 for a maximum of 80 minutes.

Alternatively, chemiluminescence was measured by a 1251 Luminometer (Bio-Orbit). Measurements were performed at 150 seconds intervals for a maximum of 60 minutes at 37° C. PMA was added after the first reading.

Western blot analysis on cellular membrane preparations.

Western blot analysis was performed on membrane preparations from lymphoblastoid cell lines. 10^8 cells were washed in phosphate-buffered saline and resuspended in 1 ml of 6% (w/v) sucrose in 10 mM Pipes, pH 7.0, with the addition of 1 µl of leupeptin (10 µg/ml) and 1 µl DFP (diisopropyl fluoro-phosphate) (1 M). After 15 min on ice, cells were lysed by two cycles of sonication of 7 and 5 sec respectively, and the suspension was centrifuged at 2000 rpm for 3 min, at 4°C. Five-hundred ml of 34% sucrose-2mM 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 membrane bands were recovered, diluted with cold 9% sucrose and centrifuged at 230,000 g (r max) for 20 min, at 4°C. The pellets were resuspended in 100 ml 9% sucrose in 10 mM Pipes, 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 the samples analyzed for gp91-*phox*, N-linked oligosaccharides were removed with endoglycosidase F according to the manufacturer's recommendation. SDS-polyacrylamide gel electrophoresis followed by immunoblotting with monoclonal antibodies MoAb 449 and MoAb 48, directed against gp91-*phox* and p22-*phox* respectively, was performed as described by Verhoven et al. (Verhoeven et al., 1989).

Southern and Northern blot analysis.

Southern blotting was performed with DNA samples extracted from 2×10^6 transduced B-cell clones and digested to completion with the restriction enzymes *Kpn* I and *Xba* I for the analysis of the integrated provirus. 20 µg of each sample were resolved by 0.8% agarose gel electrophoresis and blotted to 0.45 µm pore-size nylon membranes according to standard procedures (Sambrook et al., 1989).
Total cellular RNA was extracted from 2×10^6 transduced B-cells clones according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). For Northern blotting experiments, 20-µg samples were resolved by electrophoresis in 1% agarose gels in the presence of formaldehyde, and transferred to a 0.45 µm pore-size nylon membranes (Sambrook et al., 1989).
Both Southern and Northern blots were hybridized to single stranded DNA probes, labeled with (α^{32}P)-dCTP by the random priming technique (Sambrook et al., 1989). Hybridization conditions were as described (Sambrook et al., 1989), 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.

The following probes were used for hybridization: for gp91-*phox* gene, a *Pst* I-*Sac* I fragment of the gp91-*phox* cDNA, containing most of the coding region, derived from the pBsII-KS plasmid (Stratagene, la Jolla, CA, USA) (see above); for the β-actin probe, a 226 bp PCR amplification product obtained by amplification with the BA1 and BA4 primers (Table VIII); for the pBabeHygro/gp91-*phox* proviral DNA, a *Cla* I-*Hind* III fragment from plasmid pBabeHygro encompassing the hygromycin gene. All the probes were purified from gels after electrophoresis.

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-lymphoblastoid cell lines from the patients and amplified by PCR using the top three primer pairs reported in Table VII. Three overlapping fragments were obtained for each patient. Conditions for reverse transcription 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 CGD11 and CGD12). Polymerase chain reaction was performed in a Perkin-Elmer thermal cycler according to standard procedures. The primers used for cDNA amplification cloning and sequencing are described in Table VII.
The amplified fragments were eluted from polyacrylamide gels and directly cloned in the pCR™II vector of Invitrogen. Sequence analyses 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 (α^{35}S)-dATP. Sequence data were obtained by extension of the universal primer of the vector.
To avoid misinterpretations due to mutations introduced by Taq polymerase misincorporation during the PCR amplification process, each mutation found was verified. When the mutation affected a restriction site, a restriction analysis was performed on a newly amplified fragment obtained from a new reverse transcription reaction or, possibly, on a PCR product obtained by amplification of genomic DNA. When restriction analysis was not possible, a new fragment was cloned in the pCR™II vector and the sequence was analyzed.

primer	**sequenza nucleotidica**	**posizione** (mappa cDNA)
CGD 1	20nt 5'-TGAATGAGGGGCTCTCCATT-3'	nt 32-51
CGD B	26nt 5'-GTACAATTCGTTCAGCTCCATGGATTG-3'	nt 675-700
CGD A	25nt 5'-CCGGAGGTCTTACTTTGAAGTCTTT-3'	nt 606-630
CGD DII	20nt 5'-GCAAACCACTCAAAGGCATG-3'	nt 1360-1379
CGD C	25nt 5'-GGTGATGTTAGTGGGAGCAGGGATT-3'	nt 1224-1248
CGD 8	20nt 5'-GTAAAAGTGCTCTCAAAACC-3'	nt 1913-1932
GP915'	40nt 5'-TTAGGATCCGGTACCTCGAGCCACCATGGGGAACTGGGCT-3	(see text)
GP913'	20nt 5'-ATGCAGTTGAAATTCAGGCA-3'	nt 190-209
		posizione (mappa genomica)
CGD 11	20nt 5'-GCATAGTATAGAAGAAAGGC-3'x	nt -36/-16
CGD 12	18nt 5'-TGGTACTTACAATGACAA-3'	nt 3-20

TABLE VII. Primers used for cDNA and genomic DNA amplification, cloning and sequencing. Numbering is referred to the transcription start site of gp91-*phox* mRNA.

4. CONSTRUCTION OF RECOMBINANT RETROVIRAL VECTORS AND ESTABLISHMENT OF MURINE PRODUCER CELL LINES

Construction of pBabeHygro/gp91-phox

A plasmid containing 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 (~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 sequences are reported in Table VII . 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 (Morgenstern and Land, 1990) to obtain pBabeHygro/gp91-*phox*. This plasmid was introduced into the ecotropic packaging cell line ψ_2 using the calcium phosphate co-precipitation method (Sambrook et al., 1989) and selection was applied by addition of 150 µg/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 150 µg/ml of hygromycin B. Resistant colonies were isolated by ring cloning and expanded. The titer of produced recombinant retrovirus was tested by colony assay on NIH3T3 cells (Miller and Rosman, 1989). The clone producing the highest titer (~1 x 10^4 cfu/ml calculated with the NIH3T3 assay, see below) was used as a source of virus for transduction of B-lymphoblastoid cell lines from the patients.

Construction of pBabeHygro/TK/gp91-phox and pBabeHygro/CMV/gp91-phox

The cytomegalovirus immediate early promoter (CMV IE) and the herpes simplex virus-1 thymidine kinase promoter (HSV-1 TK) were inserted upstream the gp91-*phox* coding region, to drive the transcription of the gene. Restriction sites were inserted flanking the two promoters using PCR with tailed primers, and the two fragments were amplified using as a template the plasmid pCEP4 (Invitrogen). The CMV IE promoter was cloned as a *Bam*HI-*Eco*RI fragment in pBabeHygro/gp91-*phox*, while the TK promoter was cloned in the *Bam*HI-*Sna*BI sites of the same vectors. The vectors obtained were called pBabeHygro/TK/gp91-*phox* and pBabeHygro/CMV/gp91-*phox*. Production of the virus was performed as described for the pBabeHygro/gp91-*phox* vector

Assay for the presence of helper virus in the supernatant

The presence of helper viruses in the supernatant was tested before using the supernatant itself for infection of target cells. The supernatant taken from the packaging cells was used to infect NIH3T3 cells and the cells were selected with 200 μg/ml of hygromicin as described below. The supernatant of the selected NIH3T3 cells was used to infect other NIH3T3 cells and these cells were again selected with hygromicin. The presence of resistant colonies was determined after 14 days of selection by Giemsa staining.

4.1 Construction of the interferon-γ responsive retroviral vector pBabeHygro/IRE-TK/gp91-*phox*

An interferon-γ responsive element has been described in the promoter of the mig (monokine induced by γ-interferon) gene (Wong et al., 1994), and it is composed by an imperfect palindrome. This element is able to give interferon-γ responsiveness to a minimal TK promoter in a promyelocytic cell line (Wong et al., 1994). An element composed by a duplication of the 3' half of the palindrome was shown to have an increased activity upon interferon-γ stimulation (Wong et al., 1994).

The mutated interferon-γ element composed by a duplication of the 3'half of the palindrome (IRE-interferon responsive element) was prepared by annealing complementary oligonucleotides and ligating them into the *Bam*HI site of the pBL-TK-CAT plasmid (see below). The sequences of the complementary oligonucleotides are:

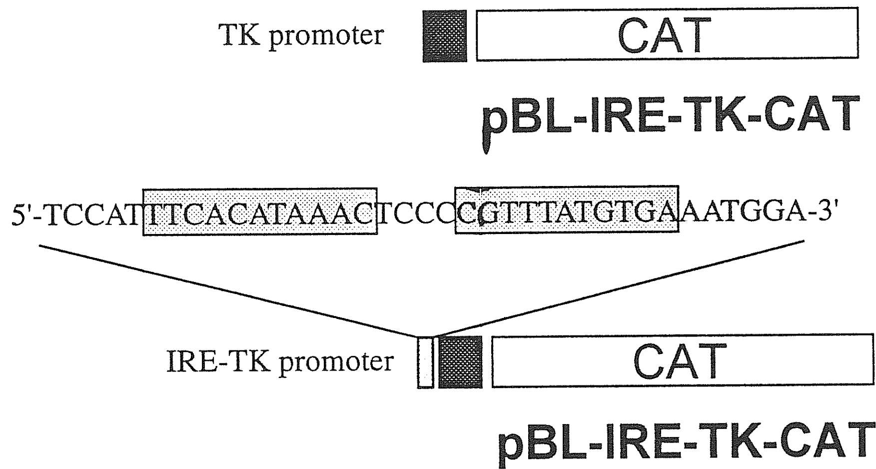
5'-GATCCAT**TTCACATAAAC**tcccc**GTTTATGTGAA**ATG-3' and

5'-GATCCAT**TTCACATAAAC**gggga**GTTTATGTGAA**ATG-3'

The oligonucleotides were designed to allow the creation of a *Bam*HI site flanking the palindrome after annealing. The generated fragment was then phosphorylated according to standard procedures (Sambrook et al., 1989) and ligated into the *Bam*HI site of pBabeHygro/TK/gp91-*phox*.

Construction of the interferon-γ responsive plasmid pBL-IRE-TK-CAT

In order to evaluate the IFN-γ inducibility of the complete TK promoter linked to the IRE element, we constructed the pBL-TK-CAT plasmid. The TK promoter was obtained as a PCR product from the plasmid pCEP4 (Invitrogen) using the primers TKP5'*Xho*I(5'-AATCACTCTCGAGGGATCTGCGGCACGCTG-3') and TKP3'*Bam*HI (3'-CCTACTTCGTCCCGTACCGCCCTAGGTATTAT-5') and cloned as a *Bam*HI-*Xho*I fragment in the plasmid pBLCAT 3 to obtain plasmid pBL-IRE-TK-CAT (see Figure 11 panel



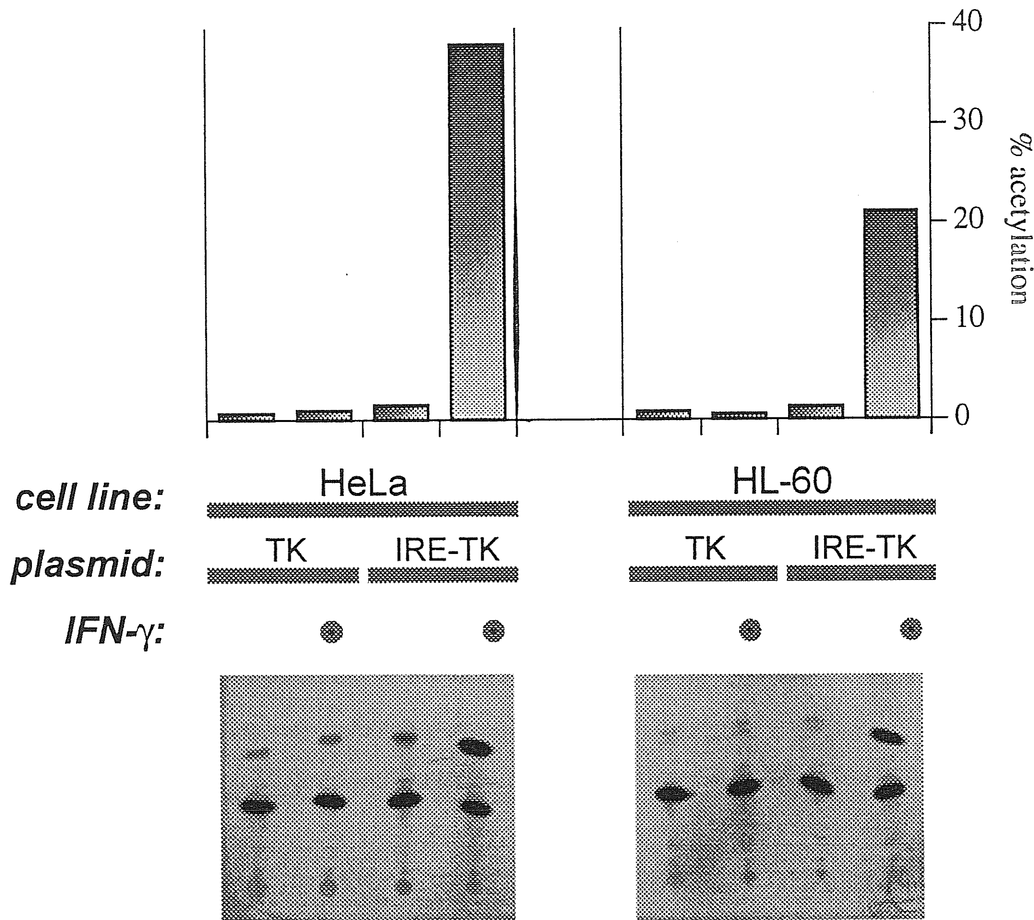


Figure 11. Activity of the IRE element in HeLa and HL60 cell lines. CAT assays were performed to detect the chloramphenicol transacetylase activity in the HeLa and HL60 cell lines. Cells were tranfected with plasmids pBL-TK-CAT and pBL-IRE-TK-CAT (panel A) and the CAT activity was determined after 24 hours of stimulation with interferon-γ. In panel (B), the results of the CAT assay are shown in both cell lines (HeLa, HL60), with both plasmids (TK, IRE-TK), with and withouth interferon-γ stimulation (IFN-γ), and without it.

These experiments have been performed at least three times obtaining consistent results; only the results of one experiment are shown.

A). The pBL-IRE-TK plasmid was obtained BY cloning the IRE element in the *Bam*HI site of the pBL-TK-CAT plasmid to obtain pBL-IRE-TK-CAT (Figure 11 panel A).

Transfection and CAT assay

Two different cell lines, HeLa and HL60, were transfected with the same amount of the two plasmids. Transfection in HeLa cells was performed according to the calcium phosphate co-precipitation procedure (Sambrook et al., 1989). $3x10^6$ cells were seeded in two 60 mm dish the day prior to transfection and the trasfection was carried out using 10 μg of each plasmid DNA. The day after, the dishes were trypsinized and one dish from each transfection group received medium alone, one received recombinant human IFN-γ (1000 U/ml Boehringer). Cells were harvested for analysis of chloramphenicol acetyltransferase (CAT) expression after 24 hours of stimulation.

Transfection for HL60 cells was performed by a modification of the DEAE-dextran method (Sambrook et al., 1989). Briefly $2x10^6$ cells were plated on a 35 mm petri dish. Twenty four hours later a batch of transfection solution (TS) was freshly prepared containing 10 μg of the CAT reporter plasmid. The volume was taken in 900 μl in sterile TBS (25mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM Na_2HPO_4). After washing the cells with TBS, 300 μl of sterile DEAE-dextran (1 mg/ml in TBS) were added to TS and the resulting mix was added to the pelleted cells. After 30 minutes of incubation at 37°C, 120 μl of DMSO were added to the cells (final concentration 10%). After an incubation of 3 minutes at 37°C, the cells were washed in TBS and supplied with culture medium.

After 24 hours, the cells from each transfection were split in two Petri dishes and one dish from each transfection received 1000 U/ml rhIFN-γ. After 24 hours stimulation, the cells were harvested for CAT assay.

Preparation of cell lysates and determination of CAT activity was performed as described previously by Gorman (Gorman et al., 1982). Protein extracts were obtained by three cycles of freeze and thaw (5 minutes in dry ice followed by 5 minutes at 37°C) and a further incubation at 60°C for 5 minutes to inhibit endogenous deacetylases. After pelletting debris, protein concentration in the supernatant was determined using 5 ml of undiluted lysate in Bradford assay (Sambrook et al., 1989). 60 μg of protein extract were used to determine CAT activities.

Samples were then incubated for 1 hour at 37°C with 2.5 μl of ^{14}C-1-deoxychloramphenicol (50 mCi/mmol Amersham), 70 μl 1M Tris-HCl, pH 7.8, 10 μl 8 mM acetyl-CoA in a final volume of 140 μl. The reaction was stopped by extracting chloramphenicol with 1 ml of ethyl acetate and, following lyophilization, samples were resuspended, spotted on TLC silica gel plates and developed by ascending chromatography in a 95% chloroform-5% methanol mixture.

Relative CAT activity was determined as a ratio between the value for the non stimulated cells and the value for the IFN-γ stimulated cells for both cell lines.

5. Isolation and transduction of peripheral blood and bone marrow hematopoietic precursors

Peripheral blood mononuclear cells were isolated from 10 ml of peripheral blood by centrifugation through standard Ficoll-Hypaque discontinuous gradients. The same procedure was also used to obtain the light density cellular fraction from frozen bone marrow buffy coat samples.

Enrichment for $CD34^+$ hematopoietic precursors was obtained using the Ceprate LC stem cell concentrator (CellPro, Wezembeek-Oppem, Belgium) according to the manufacturer's instructions.

Total mononuclear cells or $CD34^+$ precursors were washed with HBSS supplemented with 5% fetal calf serum, resuspended at $5x10^5$/ml in IMDM supplemented with 20% fetal calf serum with the addition of rhIL3 (10 ng/ml), rhIL6 (10 ng/ml), rhGM-CSF (10 ng/ml), rhSCF (50 ng/ml), and then incubated for 24 hours in a humidified CO_2 incubator at 37°C. A fraction of cells ($1x10^6$ to $2x10^6$ cells) was then collected by centrifugation and resuspended in 2 ml of conditioned medium from the PA317 producer cell line in the presence of the same growth factor mixture as above with the addition of polybrene at 6 µg/ml. Infection was carried out for 72 hours. During this period, the retrovirus-containing medium was changed every 24 hours in order to provide fresh retroviral particles.

5.1 Methylcellulose clonogenic assay.

At the end of retroviral infection procedure, cells were collected, washed in HBSS with the addition of 5% FCS and incubated in methylcellulose medium for the clonogenic assay as described (Metcalf, 1977). Briefly, $1.5x10^5$ mononuclear cells or 10^4 $CD34^+$ cells, were diluted in 1.25 ml of IMDM containing 0.9% methylcellulose, 30% FCS, 3% BSA, 10^{-5} M b-mercaptoethanol, rhIL3, rhSCF, rhGM-CSF at the final concentrations reported above, and rhEPO at 2U/ml. All assays were performed in duplicate in 35 mm Petri culture dishes. Nontransduced control cells were subjected to the same manipulations as infected cells.

Fourteen and 20 days after plating, colonies were examined, counted with an inverted light microscope, and scored according to established criteria (Metcalf, 1977).

5.2 Analysis of transduced hematopoietic cells.

At days 14 to 20 after transduction, colonies of more than 50 cells were picked up from methylcellulose under microscope guidance and washed in 100 µl of PBS. For DNA extraction, cells were placed in 50 µl of lysis buffer (Tris-HCl 10 mM, pH 7.5, $MgCl_2$ 2.5 mM, Triton X-100 0.45%, NP40 0.45%). Proteinase K was then added (6 µg/ml) and samples were incubated for 1 hour at 55°C, followed by 10 min at 95°C. Two µl were analyzed by PCR using the oligonucleotides CGD17 and SV1ter as forward and reverse primers respectively. These oligonucleotides are complementary to nt 1727-1746 in the gp91-*phox* cDNA (for CGD17) and to nt 183-202 in the early promoter region of SV40 (for SV1ter). PCR amplification with these primers generates a 206-bp product from the recombinant retroviral template. Amplification was carried out for 40 cycles according to the following parameters: 30 sec at 94°C; 30 sec at 60°C; 30 sec at 72°C. Amplification of the β-globin was performed for every sample as an internal control for the amount of DNA.

For RNA analysis, total cellular RNA from individual colonies was extracted by the guanidinium thiocyanate acid method (Chomczynski and Sacchi, 1987), reverse transcribed using the antisense SV1ter primer according to standard procedures, and then subjected to PCR amplification according to the conditions described above. In nested PCR experiments, 2 µl of the amplification reaction were amplified for further 40 cycles using primers SV1ter and CGD18X, the latter being complementary to nt 1856-1875 of the gp91-*phox* cDNA sequence. Amplification of the β-actin cDNA (primers BA1-BA4) was used as a control of the quality of the RNA preparations. All the primers used are described in Table VIII.

5.3 Superoxide production in differentiated myeloid colonies.

The NBT assay was used to evaluate O_2^- production by mature (20 days after infection) myeloid colonies in methylcellulose cultures. One ml of solution containing 0.1% of NBT and 1 µg/ml of PMA was layered over the culture. After 1 hour, cells were fixed with 1% paraformaldehyde and colonies were immediately scored for the presence of blue staining under an inverted microscope.

Cells that were grown and differentiated in liquid cultures were analyzed for superoxide production by the luminol assay according to the conditions described above.

6. Transfection of retroviral plasmids in HeLa cells

HeLa cells were transfected using the calcium phosphate co-precipitation protocol as described previously. 10 µg of each plasmid, pBabeHygro/gp91-*phox*, pBabeHygroTK/gp91-

primer	nucleotide sequence	position
SV1ter	5'-CCTAACTGACACACATTCCA-3'	nt 183-202 in pg91-*phox* cDNA
CDG17	5'-AACTTGTCTCTTCCATGAGG-3'	nt 1727-1746 in gp91-*phox* cDNA
CGD18SX	5'-ACCTGCAGGGATCCGTCGACTAAAGGAATGTCAAAGATTG-3'	nt 1856-1875 in gp91-*phox* cDNA
CGD18DX	5'-GTCGACGGATCCCTGCAGGTAGGGAAAACCATTACATTAT-3'	nt 1836-1856 in gp91-*phox* cDNA
PCO3	5'-ACACAACTGTGTTCACTAGC-3'	nt 62872-62891 in β–globin locus
PCO4	5'-CAACTTCATCCACGTTCACC-3'	nt 62981-62962 in β-globin locus
BA1	5'-CATGTGCAAGGCCGGCTTCG-3'	nt 1201-1220 in β-actin gene
BA4	5'-GAAGGTGTGGTGCCAGATTT-3'	nt 1541-1560 in β-actin gene

TABLE VIII. Oligonucleotides utilized for competitive PCR and analysis of hematopoietic progenitor colonies.

phox, pBabeHygroCMV/gp91-*phox* and pBabeHygroTK-IRE/gp91-*phox*, were used for different transfections together 2 μg of the plasmid PSV2neo. After 24 hours the cells were washed and seeded in selective medium containing 600 μg/ml of G418. After one month of selection, the cells where harvested and the RNA was extracted according to the guanidium cloride method (Chomczynski and Sacchi, 1987). The HeLa cells transfected with the pBabeHygro/TK-IRE/gp91-*phox* plasmid were stimulated for 24 hours with 1000U/ml IFN-γ prior to RNA extraction.

20 μg of the extracted RNA was analyzed by Northern blotting using the coding region of gp91-*phox* cDNA as a probe (see above). Expression of the β-actin mRNA was analyzed as an internal control.

7. Transduction of PLB-985 X-CGD cells

The promyelocytic cell line PLB-985 X-CGD, in which the gp91-*phox* gene has been disrupted by homologous recombination (Zhen et al., 1993), represent an optimal working model for gene transfer in X-CGD. To compare the activity of the different retroviral vectors in a myeloid environment, these cells were infected through coculture with the four packaging cell lines for two days in the presence of protammine 4 μg/ml. After 4 weeks of selection with 2 mg/ml of hygromicin B, cells were differentiated in RPMI medium containing di-methyl formamide at a final concentration of 0.5% (Zhen et al., 1993). After 5 days of differentiation the cells were tested for the production of superoxide by means of the luminol assay as described previously.

8. Transduction of B-lymphoblastoid cells lines

Subconfluent pBabeHygro/gp91-*phox* PA317 cells were cocultured with $5x10^5$ 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% fetal calf serum. Hygromycin B selection (200 μg/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, RNA and protein extraction and tested for superoxide production.

9. COMPETITIVE PCR FOR VIRAL DNA AND RNA QUANTITATION

The pBabeHygro/gp91-*phox* retroviral construct was used in the competitive PCR study for retrovirus titer determination. Competitive PCR was carried out with primers SV1ter s located in the SV40 early promoter and primer CGD17 in the gp91-*phox* coding region (see Figure 12 panel A). Amplification with these primers generates a 224 bp product from the viral construct template.

Construction of the competitor DNA for competitive PCR

The competitor DNA fragment was obtained as already detailed (Diviacco et al., 1992; Grassi et al., 1994). Briefly, two separate amplifications were carried out, using primers CGD18SX and SV1ter, and primers CGD17 and CGD18DX. In Figure 12 panel B, the black arrows indicate primers CGD17 and SV1ter, used for amplification; the gray arrows shows primers CGD18SX and CGD18DX, used for competitor construction. The 3' end of the last two primers correspond to contiguous sequences within the amplification target segment on opposite strands, while their 5' ends contain two 20 nt sequences unrelated to the target and complementary one each other. The two amplification products that contain a single overlapping region of 20 bp, were resolved on a 5% polyacrylamide gel and stained with ethidium bromide. The bands were eluted in 100 µl of water for 1 hour at 37°C and 1 µl of each elution was included in a standard 50 µl PCR amplification mixture containing the two outside primers (SV1ter and CGD17). To allow the formation of an heteroduplex product annealed at the complementary sequences with 5' protruding ends (the only product which could be further extended and amplified), the reaction was denatured at 94°C for 3 minutes and then the temperature was slowly lowered to 50°C within 10 min. After further 2 minutes 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: 30 cycles; 94°C 30 seconds; 56°C 30 seconds; 72°C 30 seconds. The amplification product obtained by this recombinant PCR technique has exactly the same sequence as the corresponding portion of the cDNA of the starting template except for the 20 bp insertion in the middle. This fragment was quantified against the pBabeHygro/gp91-*phox* plasmid and directly used as a competitor.

The location and the sequence of the primers used for PCR amplification and construction of the competitor are shown in table VIII.

Viral DNA and RNA quantification by competitive PCR

Competitive PCR was applied to measure the concentration of viral genomes in packaging cell clones supernatants and proviral DNA formed upon infection of different cell

lines. For the former measurement, RNA was extracted from 500 µl of supernatant by the guanidinium thiocianate method (Chomczynski and Sacchi, 1987) and the cDNA was obtained by reverse transcription of 1/5 of total extracted RNA as described (Grassi et al., 1994), using the SV1ter primer.

Briefly, 3 µl of RNA out of 15 µl were reverse transcribed in 50 µM 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 (GIBCO BRL, Gaithersburg, MD) (final reaction volume 20 µl). RNA and primers were annealed for 8 minutes at 65°C in reaction buffer and RNasin and then cooled on ice before addition of nucleotides and enzyme. The reaction mixture was allowed to proceed for 45 minutes at 37°C. The reaction was stopped by heating at 95°C for 5 minutes. Five µl of the RT reaction was then used in a 100 µl PCR reaction.

To calculate the retroviral titer by quantitation of proviral DNA molecules formed in the target cells, cell cultures were infected and 24 hours after infection cells were trypsinized, pelleted, and resuspended in 200 µl of lysis buffer (proteinase K 60 mg/ml, $MgCl_2$ 2.5 mM, Tris-HCl pH 8.0 10 mM, Nonidet-P40 0.45%, Tween-20 0.45% in Perkin Elmer PCR buffer 1x). After 1 hour incubation at 55°C, proteinase K was heat-inactivated by incubation at 95°C for 15 min, cell debris was pelleted, and 2 µl of the supernatant were used for the competitive PCR reactions.

Competitive PCR was carried out on sample DNA with the addition of increasing 10-fold concentrations of competitor, in 100 µl of PCR buffer (50 mM KCl, 10 mM Tris-HCl, 2 mM $MgCl_2$) containing the two primers (100 pmol each), the four dNTPs (200 µM each) and 2.5 U of *Taq* DNA polymerase (Perkin Elmer Emmeryville, CA). Samples were submitted to 50 cycles of amplification with the following cycle profile: denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec.

NIH3T3 colony assay

For the NIH3T3 colony assay (Miller and Rosman, 1989), $2x10^5$ recipient NIH3T3 cells were seeded on 60 mm dishes. After 24 hours, each culture was fed with 2 ml of fresh medium containing 8 µg/ml polybrene and 100 µl of viral supernatant from a PA317 cell clone. After 6 hour incubation, the medium was changed and after additional 24 hours the cells were seeded on 10 mm dishes in the presence of 150 µg/ml of hygromicin B (Sigma, St. Louis, Missouri). After 12 days of selection, cells were washed with PBS, incubated for 15 minutes in PBS plus 5% formaldehyde, washed with water and stained with Giemsa 5% in water. Virus titer was calculated according to the number of visible colonies. Given the expected variability of this assay (Lynch and Miller, 1991), each determination was performed in duplicate, and the mean colony number was considered.

Provirus copy number determination after cell infection

A time course experiment was performed by transduction of $2x10^5$ murine NIH3T3, $2x10^6$ human HeLa and $1.5x10^6$ human 293 recipient cells, seeded on 60 mm dishes. Supernatants from two different producer clones (clones 36 and 52 of pBabeHygro/gp91-*phox*) were used for transduction. At 24 hours after infections, cells were seeded on 10 mm dishes and then harvested at days 1, 7, 14 post infection (HeLa and 293) or days 1, 7, 14 and 21 (NIH3T3) for provirus copy number determination by competitive PCR.

For the colony assays, cells were selected in the presence of 150 µg/ml (HeLa and NIH3T3) or 250 µg/ml (293) of hygromicin B. Titers were estimated from the number of colonies after 12 days of selection.

Both PCR and colony assay titers are expressed per ml of packaging cell clone supernatant.

Results

Section A

1. CHARACTERIZATION OF THE GENETIC DEFECT IN THREE PATIENTS WITH X-LINKED CGD

Since 1995, the characterization of the genetic defects responsible for X-CGD was initiated to be performed in the Italian population, within the framework of a nationwide Italian collaborative group. Aims of this activity are: i) the definition of common diagnostic and therapeutic protocols; ii) the characterization of mutations responsible for the disease; iii) the enrollment of patients in preclinical and clinical (in the future) studies of gene therapy.

Currently, samples from over 25 families with CGD have been analyzed for mutations. In this thesis, I will describe the characterization of the genetic defect of three children with X91^{o} CGD. Samples from these 3 patients have been used in the gene transfer studies described in the following sessions. The three patients A.G., N.B. and A.Z. are affected by serious bacterial infections with slow resolution from a very early age (in particular, osteomyelitis of long bones, suppurative pneumonia and lymphadenitis caused by coagulase positive *St aureus*, *S. marcescens* and *Candida*. Laboratory diagnostic criteria were the complete absence of reduction of nitro blue tetrazolium by granulocytes in the NBT-slide test and the complete absence of oxidase production after incubation with zymosan (at least four tests were performed for each patient). Patient N.B. died in 1996. Peripheral blood progenitor cells, lymphocytes, and bone marrow samples were obtained from these three patient after informed consent.

A set of assays were performed to characterize the genetic defect responsible for the disease.

To analyze the functionality of the NADPH oxidase in the three linphoblastoid cell lines derived from the patients, superoxide production was analyzed by a luminol-based chemiluminescent assay. Subsequently, the presence of cytochrome b_{558} proteins was investigated using antibodies against gp91-*phox* and p22-*phox* .

To verify the possibility that the disease was caused by gross deletions and DNA rearrangements, the DNA of the three patients was analyzed by Southern blotting using the respective lymphoblastoid cell lines as a source of genomic DNA. As a second step in DNA analysis, the sequence of the complete gp91-*phox* coding region was determined to detect possible point mutations. Finally, the transcriptional pattern of gp91-*phox* was analyzed by Northern blotting in the three patients.

1.1 Establishment of lymphoblastoid cell lines

Normal lymphoblastoid cell lines express a small amount of functional cytochrome b_{558} on their membrane (Volkman et al., 1984). The NADPH complex appears to be the same as the one produced in phagocytes, although its level is reduced to 1-10%. Importantly, B-cell lines from CGD patients show the same oxidase disfunctions as those found in phagocytes from the same patients (Porter et al., 1992; Volkman et al., 1984). For these reasons CGD B cell lines are excellent cellular models of the NADPH oxidase, and 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 a source of genomic DNA, messenger RNA and cellular membranes. Additionally lymphoblastoid cell lines are also a good in vitro model for the development of strategies for gene therapy of CGD since they constitute a very useful tool for monitoring gene expression and functional reconstitution of oxidase activity after transduction with expression vectors bearing the gene of interest.

The supernatant of the lymphoblastoid marmoset cell line B95-8 was used as a source of infectious Epstein Barr virus (EBV) for immortalization. This supernatant is added to purified mononuclear cells to infect them with the virus and the activation of reactive T lymphocyte clones against the EBV-infected cells is avoided using cyclosporin A (Neitzel, 1986).

After infection of the B cells, two distinct morphologic features can be observed within few days. First, blastogenesis becomes evident resulting in enlargement of the infected lymphocytes and, second, there is an increasing development of cell clusters of proliferative lymphoblastoid cells. This is the result of a higher expression of adhesion proteins in transformed lymphocytes (Vyth-Dreese et al., 1995).

1.2 Analysis of superoxide production

The functionality if the NADPH oxidase complex in intact cells was assessed using a very sensitive chemiluminescent test, that records the instantaneous level of H_2O_2 formed by dismutation of O_2^- (Wymann et al., 1987). The basis of this assay is the oxidation of luminol by peroxidase (which in the case of neutrophils may be myeloperoxidase) to form the activated aminophtalate anion, that is luminescent. Owing to the fact that lymphoblastoid cell lines do not possess myeloperoxidase activity (Porter et al., 1992), horse-radish peroxidase was added to the components of the assay. Phorbol-12-myristate-13-acetate (PMA) was used to trigger superoxide generation by activating 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.

For each lymphoblastoid cell line (from patients and healthy donors), 10^6 cells were tested before and after stimulation with PMA. Chemiluminescence measurements were taken at 10-minutes intervals from time 0 (absence of stimulation) to time 80'. The data obtained by the analysis of 12 B-cell lines established from normal individuals are reported in Figure 3 panel A. From the data obtained, it is evident that the oxidase burst is strictly PMA-dependent, with a maximum of product generated at about 40 min after addition of PMA to the cells. From this time on, the chemiluminescence intensity decreases progressively for all the samples, although at different rates. A great clonal variability in the maximum amount of superoxide produced was detected among the normal cell lines analyzed, ranging between 6.4 x 10^6 cpm (sample N8) to 1.2 x 10^6 cpm (sample N7). This variability is likely to reflect biological differences among the individual B-lymphoblastoid cell clones.

For all the three lymphoblastoid cell lines of the patients (Figure 3 panel B), no increase of chemiluminescence intensity could be detected after PMA stimulation, thus demonstrating the complete inability of patients' cell lines to produce appreciable levels of superoxide. The three patients, lacking the gp91-*phox* protein as seen in the western blotting analysis, and having 0% of normal oxidase activity, can be classified within the $X91^o$ CGD subtype, which is the most common (50%) and in general the most severe subtype.

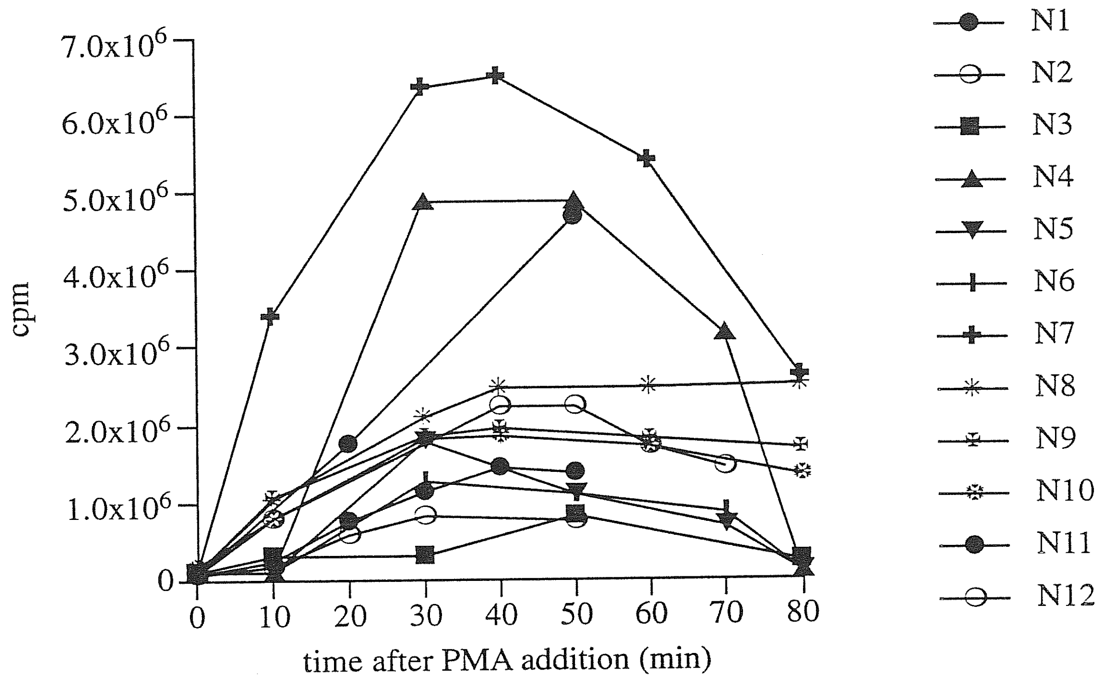
1.3 Western blotting

To analyze the presence and the properties of the cytochrome b_{558} in these patients, the cell membrane of lymphoblastoid cell lines of the patients as well the peripheral blood granulocytes were used for Western blotting analysis. Two monoclonal antibodies (MoAb 48 and MoAb 449) raised against the gp91-*phox* protein and p22-*phox* protein respectively (Verhoeven et al., 1989) were used. The antibody MoAb 48 binds only detergent-solubilized cytocrome b_{558} in the neutrophil membrane, while MoAb 449 can bind to permeabilized cells.

Lymphoblastoid cell line membranes from the three patients and from normal controls, were prepared from 10^8 cells. An aliquot of each membrane extract was treated with endoglycosidase F which cleaves the N-linked oligosaccharides from the gp91-*phox* protein. With these experiments, it was possible to detect both the glycosylated form of gp91-*phox* protein and the core protein (Figure 4 panel A). The glycosylated form of the protein typically appears as a smear from 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.

The western blotting analysis performed on granulocytes and lymphoblastoid cell lines showed, as expected (Cohen-Tanugi et al., 1991), that the amount of gp91-*phox* protein was roughly 10 times less than that found in granulocytes (data not shown).

A



B

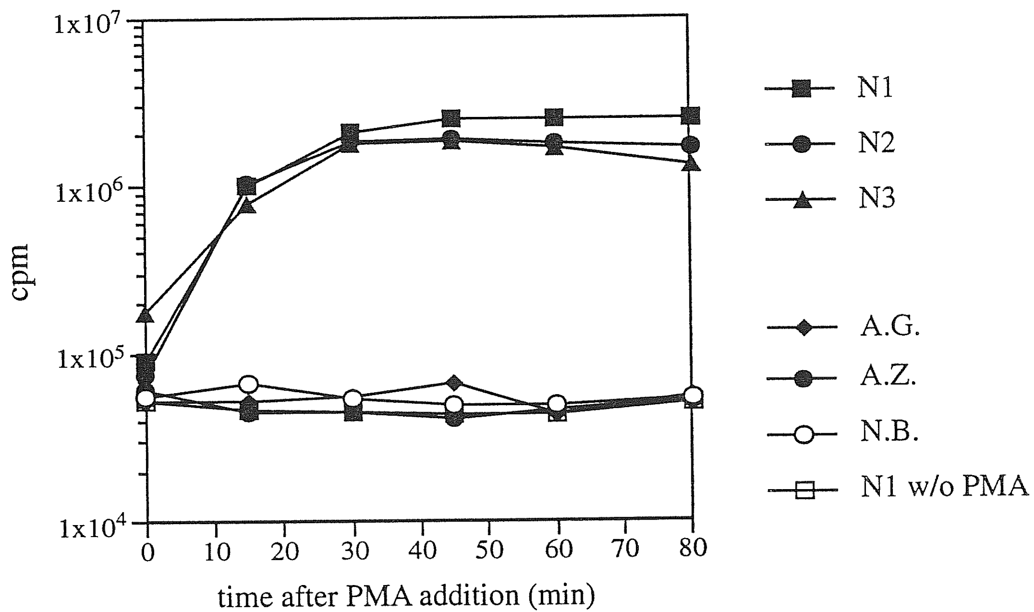


Figure 3. Luminol chemiluminescence assay of lymphoblastoid cell lines of three X-CGD patients and normal controls.

(A) Respiratory burst in 12 lymphoblastoid cell lines established from normal individuals (N1-N12)

(B) Absence of oxidase activity in the cell lines from patients A.G., A.Z. and N.B. compared to three normal controls (logarithmic scale).

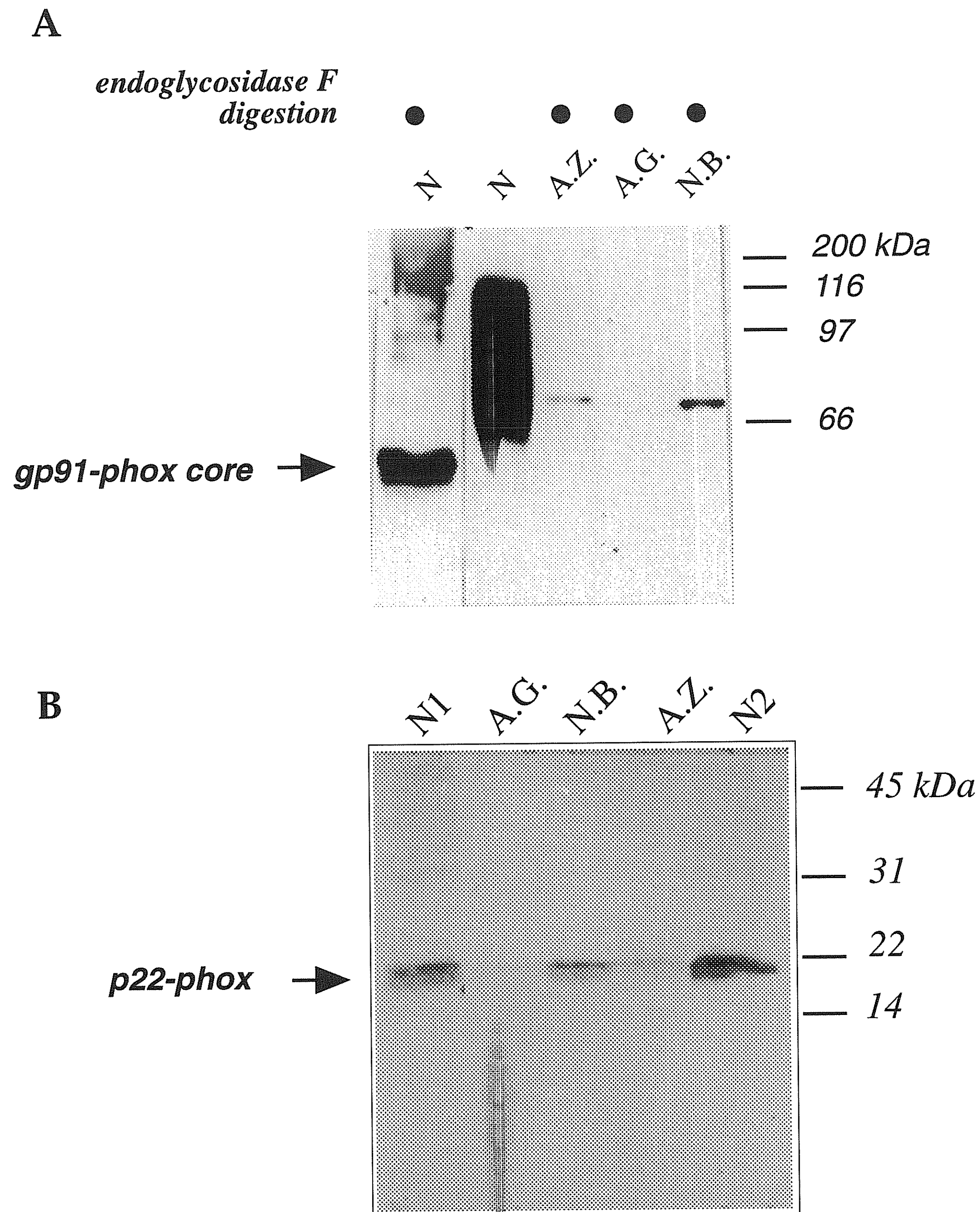


Figure 4. Western blotting analysis of the membrane proteins of the NADPH oxidase complex in the B-cell lines of the patients and of the normal controls. (A) The analysis of the gp91-*phox* protein was performed using the monoclonal antibody MoAb 48; in a normal control this Ab detects both the glycosilated and the deglycosilated forms of the protein in the membrane preparation (N; 50 µg). No gp91-*phox* protein is detectable in the membrane of the three patients (A.Z., A.G. and B.N.; 50 µg of membrane preparation each).
(B) The monoclonal antibody MoAb 449 was used to detect the p22-*phox* protein in the membrane preparations. A faint band corresponding to the 22 kDa protein is revealed in the three patients (A.Z. 100 µg, A.G. 50 µg and N.B. 100 µg). N1 and N2 are normal controls (50 and 60 µg respectively).

The experiment carried out using membranes from lymphoblastoid cell lines of the patients and of healthy individuals demonstrated that no gp91-*phox* protein was detectable in protein samples from CGD patients, while the protein was detected in the normal control (Figure 4 panel A). The bands visible in the patients A.Z. and N.B. are probably due to nonspecific staining. This observation is supported by the fact that in the patient A.Z. the gp91-*phox* mRNA is about 100 times less abundant that in the other two patients (Grassi, PhD Thesis). Morover in patient N.B. a point mutation leads to a stop codon at position 21 (see below). For these reasons the presence of any form of the protein in these two patients is improbable. We also 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}. This analysis revealed the presence of p22-*phox* in all the patients, although at different levels (Figure 4 panel B) . This is rather unusual, since in the majority of the examined cases of X91° CGD, both subunits are usually absent (Parkos et al., 1989). However, a few other cases are described in which p22-*phox* is stable in the absence of gp91-*phox* (Bolscher et al., 1991; Verhoeven et al., 1989). To further investigate this point we repeated the western blot experiment using another polyclonal antibody (a kind gift of R. Garcia, ICGEB Trieste). This experiment confirmed the data obtained by the monoclonal antibody MoAb 449. Further investigations will be required to better clarify this point.

1.4 Genomic DNA analysis

Southern blotting

Gross deletion in the gp91-*phox* gene have been found so far in 2 of the 25 Italian families analyzed (8%) (unpublished results). The percentage is similar to the percentage detected by the international study group (10%) (Roos et al., 1996). The defects in the gp91-*phox* gene in these cases are associated with large deletion of the X chromosome resulting in the McLeod syndrome. To evaluate the presence of gross deletion within the gp91-*phox* gene, the DNA of the three patients was analyzed by means of Southern blotting. 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 5 DNA samples from the three patients (A.Z., A.G., N.B.) and samples from normal individuals (normal controls) were digested with *Hind* III, *Nsi* I and *Eco* RI, the resulting fragments resolved by gel electrophoresis, transferred to a nylon membrane and hybridized. It is apparent from Figure 5 that the hybridization patterns for *Hind* III and *Eco* RI enzymes are indistinguishable for either the patients and the normal individuals. The hybridization pattern for *Nsi*I detects two sets of polymorphic 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 (not shown in the figure) respectively). The presence of these polymorphisms has been already reported (Pelham et al., 1990).

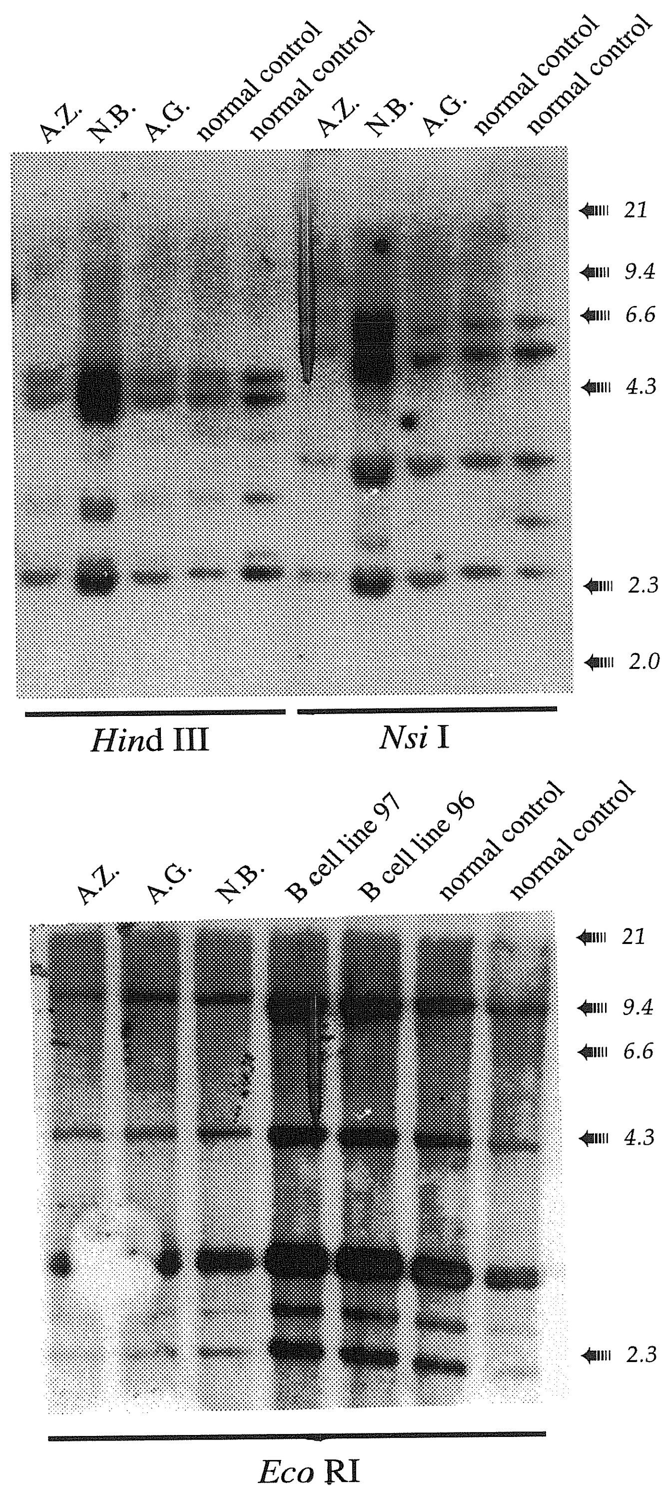


Figure 5. Southern blotting of the lymphoblastoid cell lines of the patients and of two normal controls.
DNA extracted form the B-cell lines from the three patients (A.Z., A.G. and N.B.) and from normal controls were digested with Eco RI, Hind III and Nsi I as indicated, and analyzed by Southern blotting using a fragment encompassing the coding sequence of gp91-*phox* cDNA as a probe. The Nsi I enzyme detected two DNA polymorphisms (of 2.9 or 2.5 kb and 1.7 or 1.3 kb respectively - not shown in the Figure).

As stated above, only a portion of the cDNA (corresponding to the coding region of ~1.9 kb) was used as hybridization probe. As a consequence, only a portion of the gp91-*phox* gene, totally spanning about 30 kb (Roos, 1994), was explored by this hybridization. Nevertheless, gross deletions of the genomic locus or selective deletions of the coding sequence of the gene were excluded in these patients.

Sequence analysis

A high percentage of mutations causing X-CGD involves point mutations, such as small deletions, missense mutations, nonsense mutations and splice site mutations. Therefore, to analyze the lesion responsible of the CGD phenotype of these patients, the entire coding region of the gp91-*phox* cDNA was sequenced. To facilitate cloning and sequencing the cDNA was obtained from the mRNA by PCR in five overlapping segments as detailed in material and methods section; the different fragments were cloned in the pCRTM II vector.

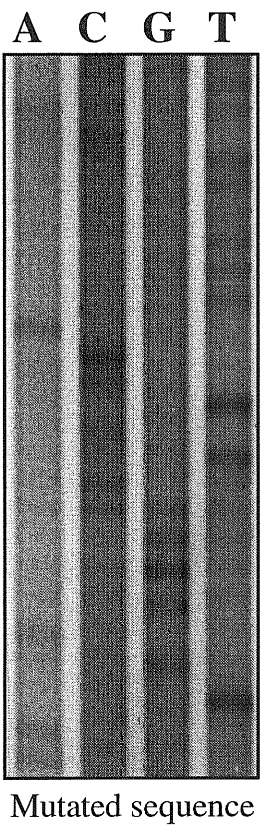
Sequence analysis showed the presence of different point mutations in the three patients Figure 6 shows the mutations found in two of the three patients. In patients A.G. there is an insertion of a T at nucleotide position 404 inside the exon 5 coding sequence; this insertion predicts an early stop codon at position 133 and a frameshift. The sequence derived from patient A.Z. reveals deletion of an A at nucleotide 1330, within the splice acceptor site of exon 11. This mutation causes a frameshift and a stop codon at position 501. In patients N.B. the deletion of a G at position 40 causes a frameshift and a stop codon at position 21 (Figure 7).

An accurate analysis was done to exclude that the detected mutations were not produced by a misincorporation of the Taq polymerase 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. The RNA was reverse transcribed, amplified and digested with the previously mentioned enzymes. This experiment confirmed the presence of the two mutations. In patient N.B., the presence of the mutation was confirmed by sequence analysis of a newly synthesized PCR fragment, obtained from a new product of reverse transcription after cloning in the pCRTM II vector. Also in this case, the mutation was confirmed.The mutations detected in these three patients predict frame shift and consequently stop codons leading to premature termination of the mutated proteins.

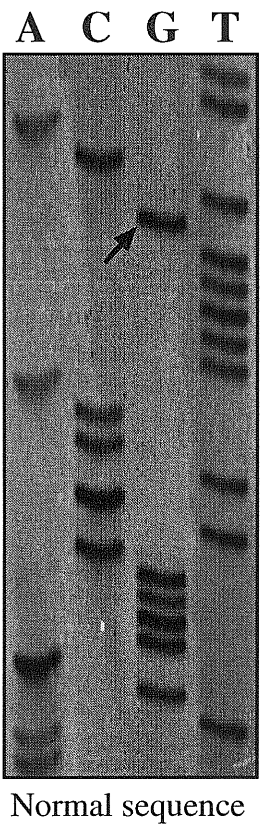
1.5 Northern blotting

The transcriptional pattern of gp91-*phox* was analyzed by Northern blotting in the three patients, to verify whether the detected mutations could somehow affect transcription or stability of the gp91-*phox* mRNA.

Patient N.B.

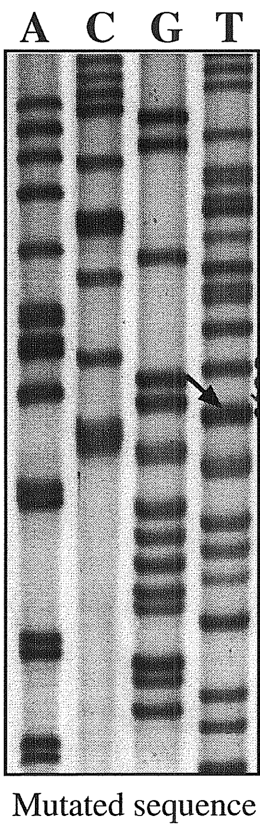


Mutated sequence

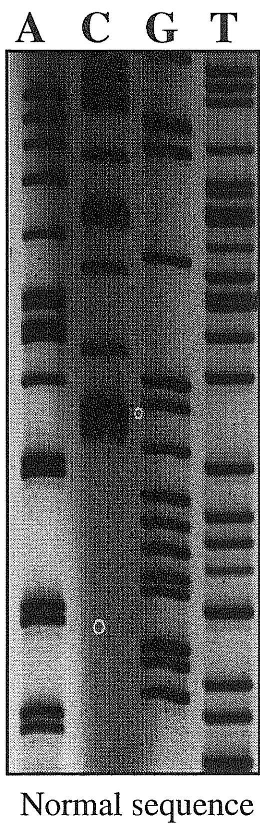


Normal sequence

Patient A.G.



Mutated sequence



Normal sequence

Figure 6. Sequence analysis of the gp91-*phox* cDNA of two patients. Mutations are indicated by arrows.

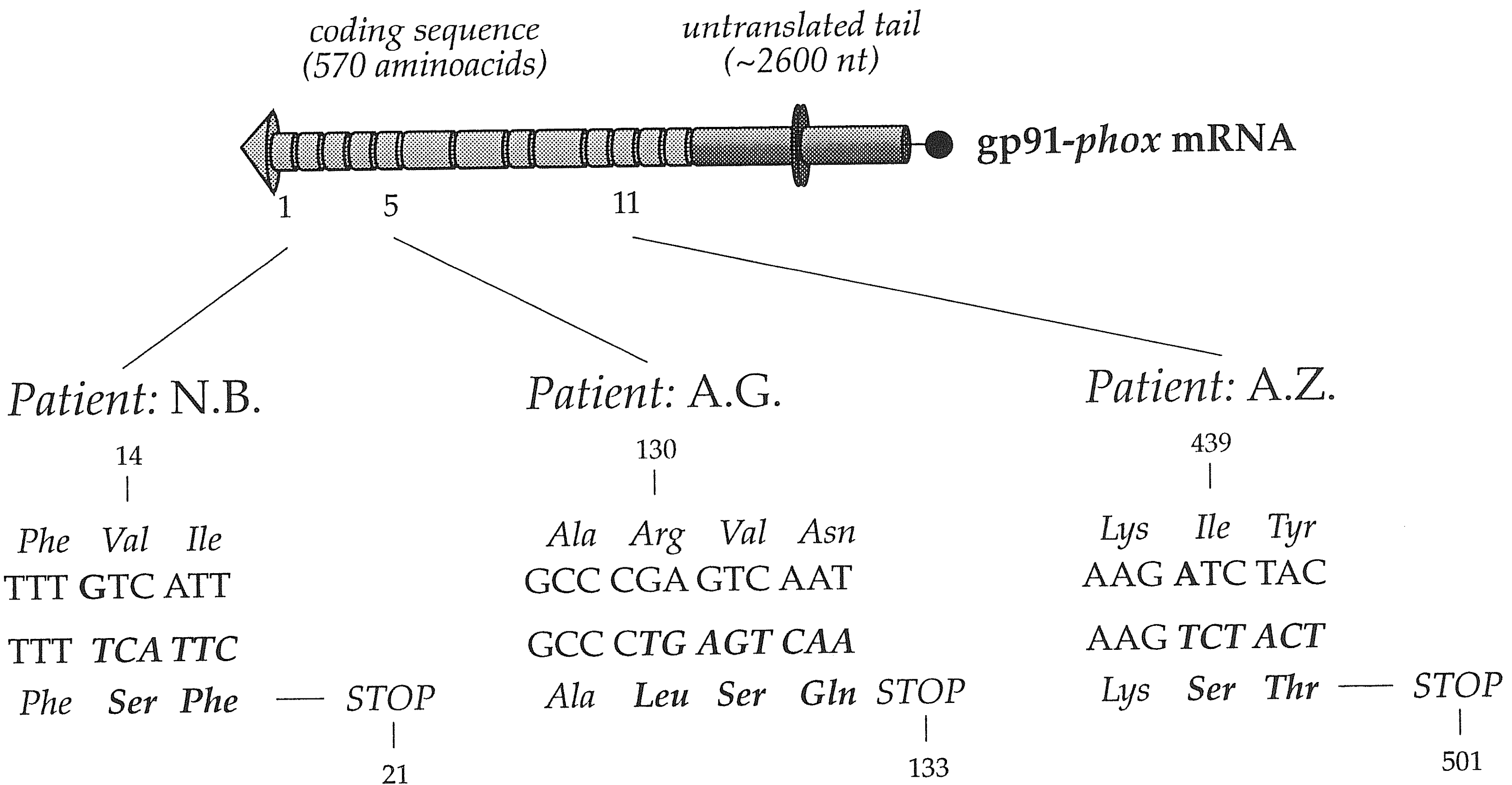


Figure 7. Mutations of the gp91-*phox cDNA*.
The analysis of the sequence of the cDNA in the three patients revealed the presence of single point mutations. Point deletions in N.B. and A.Z. and one base insertion in A.G. lead to frameshift and insertion of premature STOP codons, as indicated. The normal sequence is shown in the upper part; out-of frame sequences are typed in bold letters. Numbering refers to the aminoacid sequence.

Total RNA was extracted from the lymphoblastoid cell lines of the patients and of three normal controls; 20 μg of RNA were resolved on a denaturing agarose gel, blotted on a nylon membrane and hybridized with two different probes, corresponding to the gp91-*phox* coding region and to the β-actin mRNA, as described in the Material and Methods section. The β-actin probe was used as an internal control of integrity and amount of the RNA.

The results of the Northern blot (Figure 8) clearly show that in patients N.B. and A.G. the gp91-*phox* transcript is detectable as a band of size and amount comparable to the transcript in the normal controls. It is possible to observe also the presence of a barely detectable band migrating faster than the predominant transcript. This minor, smaller transcript is likely to be the product of an alternative start site normally found in phagocytic cells (Newburger et al., 1994).

As far as patient A.Z. is concerned, a markely reduced amount of the gp91-*phox* mRNA is detected, while the length of the transcript appears of normal size. The smaller gp91-*phox* transcript usually found in healthy controls, is present also in this sample, although very reduced in intensity.

Section B

2. Development of retroviral vectors for X-CGD gene therapy

In this section, the construction of retroviral vectors for gene therapy of X-CGD will be described. In particular I will report on: i) the construction of retroviral vectors; ii) the development of a rapid technique for retroviral titering; iii) the analysis of the properties of the developed vectors.

2.1 Retroviral constructs for gp91-*phox* gene transfer

Four different retroviral vectors were developed for gene transfer of gp91-*phox* (Figure 8). They are all based on the Moloney retroviral backbone using the pBabeHygro construct (Morgenstern and Land, 1990). This construct lacks most of the viral genes with the exception of a small portion of gag whose presence increases incapsidation efficiency (Armentano et al., 1987).

In addition to the SV40-hygro cassette that allows selection of the transduced cells, these vectors express the therapeutic gene directly from the LTR (pBabeHygro/gp91-*phox*), from strong internal promoters (pBabeHygro/TK/gp91-*phox*, pBabeHygro/CMV/gp91-*phox*) or from an IFN-γ inducible promoter (pBabeHygro/IRE-TK/gp91-*phox*).

pBabeHygro/gp91-phox

This vector is based on the pBabeHygro vector and expresses the inserted gene from the MoMuLV Long Terminal Repeat (LTR). A 1.9 kb cDNA fragment, encompassing the entire coding region of gp91-*phox* was cloned in the retroviral vector to obtain a plasmid construct. (Figure 9). With the purpose of obtaining a replication defective virus for efficient transduction of target cells, this plasmid was transfected in the ecotropic packaging cell line Ψ2 (Mann et al., 1983). This helper cell line carries integrated in its genome the trans-elements required for replication of the replication defective viral vector. The env gene integrated into its genome encodes for an envelope that will allow the retroviral vector to infect only rodent cells. Hygromycin B-resistant Ψ2 cells were selected and their supernatant used to transduce the amphotropic PA317 cell line (Miller and Rosman, 1989). This packaging cell line contains an integrated env gene that allows infection of both rodent and non rodent cell, including human cells. This procedure involving the transfection and subsequent infection of two packaging cell lines is aimed at increasing the retroviral titer. Infact the transfection procedure and the consequent integration in the cell genome is likely to randomly break the proviral construct. As a

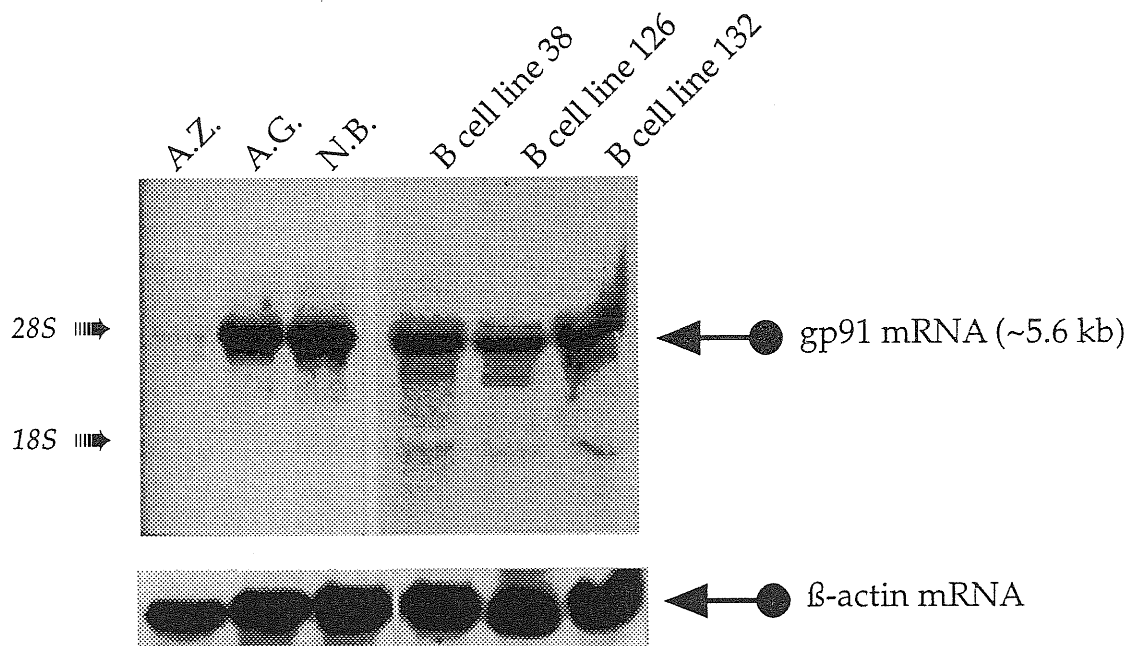


Figure 8. Northern blotting of the RNA from lymphoblastoid cell lines of the patients .
The transcriptional pattern of the gp91-*phox* gene in the three patients (N.B., A.G. and A.Z.) and in three normal controls (N1-N3) was analyzed by Northern blotting of the RNA extracted from the lymphoblastoid cell lines. A 1.9 kb fragment of the coding region was used as a probe. Subsequently, the filter was hybridized with a probe for β-actin for normalization.

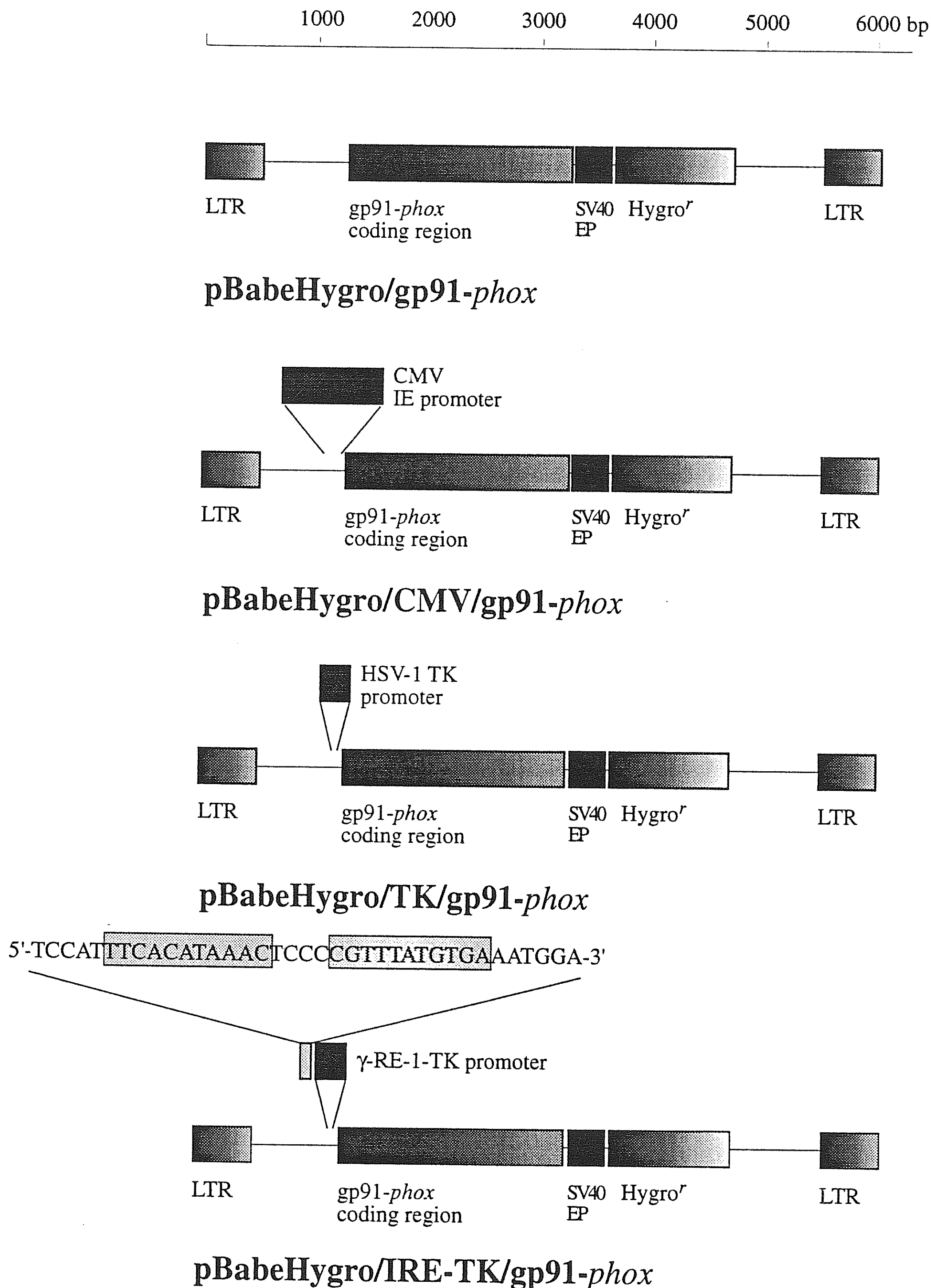


Figure 9. Schematic representation of the four retroviral vectors used in this work. From top to bottom: pBabeHygro/gp91-*phox*, pBabeHygro/CMV/gp91-*phox*, pBabeHygro/TK/gp91-*phox*, and pBabeHygro/TK-IRE/gp91-*phox*. The location of the relevant genetic elements is indicated (LTR: long terminal repeat; SV40EP: SV40 early promoter; hygro: hygromicin resistance gene; CMV IE: cytomegalovirus immediate early promoter; HSV-1 TK; Herpes simplex virus 1 thymidine kinase promoter); IRE: interferon-γ responsive element.

consequence, only few integration events result in the production of infectious viruses in the first packaging cells. The second packaging passage ensures that only the infective and complete viruses are transduced and get integrated into the chromosomal DNA.

The transduced PA317 cells were then selected with hygromicin to obtain different producing clones. Resistant colonies were isolated by ring cloning and expanded. The supernatants of 80 different clones were tested for the viral titer in order to chose the best producer for the infection of the target cells. The supernatants were assayed both by colony assay on murine fibroblast NIH3T3 cells and by the PCR procedure for viral titering as it will be described later. The supernatant from the PA317 clone producing the highest viral titer ($1x10^4$ cfu/ml in NIH3T3 colony assay and $1x10^6$ proviral molecules/ml of supernatant in competitive PCR assay) was further tested for the presence of replicative viruses on NIH3T3 cells; since no replicative virus was detected, this supernatant was used for further transductions.

pBabeHygro/TK/gp91-phox and pBabeHygro/CMV/gp91-phox

As alternatives to the viral LTR promoter element, other viral promoters were used to drive the transcription of the therapeutic gene (Figure 9). The cytomegalovirus (CMV) immediate early (IE) promoter is a very strong promoter of ~900bp, containing a polymerase II basal promoter between +7 and -65, a strong complex enhancer between -65 and -525, a modulator sequence between -750 and -1145, and a cluster of nuclear factor 1 (NF1) binding sites between -750 and -600. The promoter contains binding sites for the ATF (activating transcription factor), AP1 and NF-κB transcription factors (Ghazal et al., 1990).

In some published works (Byun et al., 1996; Hantzopoulos et al., 1992), the CMV IE promoter has been demonstrated to be stronger than the Moloney LTR in driving expression of a reporter gene, both in the context of plasmid and retroviral vectors, in all the cell lines analyzed (including erythroid K562, myeloid U937, epithelial HeLa and 293T, and fibroblastoid NIH3T3). In addition, in studies in which several promoters were compared, the CMV promoter was one of the most functional in hematopoietic cells (Malik et al., 1995). Interestingly, the activity of the CMV promoter appears to be increased in differentiated macrophage lineages, such as differentiated HL60, suggesting that the transcriptional regulation of this promoter is contributed by macrophage-specific cell factors (Malik et al., 1995). Moreover, it has been shown that the CMV IE promoter is even stronger than myelomonocytic specific promoters in differentiated HL60 cells (Malik et al., 1995). It is likely that these high levels of expression are the resultant of the presence of numerous transcription factor binding sites. In vivo experiments in mice demonstrated that the CMV promoter ensure expression of a retroviral vector-transduced gene in fibroblasts (Scharfmann et al., 1991).

The HSV-1 thymidine kinase (TK) gene promoter contains binding sites for the cellular transcription factors TFIID, Sp1 and CAAT-binding proteins, each of which affects expression of the thymidine kinase gene (Imbalzano et al., 1991). Also this promoter has been used in

many retroviral vectors as an internal promoter because of its ability to drive strong expression of the transduced genes also in hematopoietic cells (Hantzopoulos et al., 1992). In transgenic mice, expression of a retroviral vector transduced gene under the control of the TK promoter could be demonstrated in all the tissues, while other promoters like the Moloney LTR and SV40 could not (Stewart et al., 1987). This observation suggests that the suppressive mechanism affecting these promoters do not affect the activity of TK promoter.

The viruses pBabeHygro/TK/gp91-*phox* and pBabe/CMV/gp91-*phox* were produced using the packaging cell lines as previously described. During these procedure, we noticed that the hygromicin resistance of the transfected cells was markely reduced. This effect was probably due to the transcriptional interference of the CMV and TK promoters on the downstream -located SV40 promoter, driving expression of the hygromicin gene. Similar findings have been already described for other retroviral vectors containing different transcription units (Emerman and Temin, 1986; Emerman and Temin, 1986), and support the recent utilization of bicistronic strategies for the expression of two ORFs from a single transcript (Ding et al., 1996). To overcome this problem, infectious recombinant viruses were produced by a single passage in the PA317 packaging cell line. For this purpose, the plasmids containing the retroviral vectors were cotransfected with the plasmid pSV2neo directly in the packaging cells which were then selected using G418. The viral titers were determined by competitive PCR (see below) by the analysis of 50 different clones. The highest titer detected among 50 clones for each vector was of $2x10^5$ proviral molecules/ml supernatant for the CMV vector and $2.5x10^5$ proviral molecules/ml supernatant for the TK vector. These titers are 1 order of magnitude lower than those detected for pBabeHygro/gp91-*phox* (Figure 9).

pBabeHygro/IRE-TK/gp91-phox

Administration of rIFN-γ has a positive therapeutic effect on the symptoms of CGD. Infact, it enhances killing capacity of these cells with consequent reduction of infections in the treated patients (Sechler et al., 1988).

A few years ago, a large multicentre study has been carried out on 128 CGD patients, which were treated with IFN-γ (The International Chronic Granulomatous Disease Cooperative Study Group, 1991). This study demonstrated the benefits of IFN-γ treatment causing a reduction in the number of serious infections, but for most of the patients no improvement of O_2^- production or bacterial killing in vitro could be demonstrated. Morover no significant changes were observed in the mRNA levels of the oxidase components, apart from the p47-*phox* mRNA (Weening et al., 1996). Thus, IFN-γ seems to increase host defense also by enhancing non oxidative mechanisms and other aspects of phagocytic function like diapedesis and locomotion.

The interferons constitute an important group of cytokines possessing several biological properties such as antiviral activity, capacity of modulation of cell growth and differentiation, influence of the regulation of the immune response (Pestka et al., 1989). The effects of INFs are triggered through the induction of specific RNAs and proteins in responding cells (Revel and Chebath, 1986). Although type I and II IFNs bind to different receptors, they induce the expression of overlapping genes (Pestka et al., 1989). The analysis of genes activated by the IFNs led to the identification of the interferon-stimulated responsive elements (ISRE) and of the IFN-γ activation site (GAS) (Bandyopadhyay et al., 1990; Decker et al., 1991).

An IFN-γ responsive element has been isolated in the promoter of the mig (monokine induced by γ-interferon) gene (Wong et al., 1994). The mig gene encodes a member of the platelet factor 4-interleukin-8 family of low molecular weigh cytokines the transcription of which occurs upon treatment with IFN-γ.This activation is due to the presence of an IFN-γ-responsive element called γRE-1, a palindromic element that is able to confer IFN-γ responsiveness to a minimal TK promoter in CAT assay (Wong et al., 1994). The γRE-1 element has been demonstrated to bind the proteic factor γRF-1 composed by two proteins which interact with the γRE-1 site. The γRF-1 protein is present in nearly all cell types examined, such as monocytic, lymphocytic, epithelial, fibroblastoid cell lines (Guyer et al., 1995).

The imperfect palindrome responsive element has been analyzed by mutational analysis, demonstrating that a palindrome formed by a duplication of the 3' half side is even more effective in the promotion of IFN-γ responsiveness (Wong et al., 1994).

The palindrome composed by a duplication of the 3' half of the γRE-1, named by us IRE (interferon-γ responsive element), was assayed for its ability to confer responsiveness to a complete TK promoter via transient transfection and CAT assay in HeLa and HL60 cell lines. The plasmids used were pBL-TK-CAT and pBL-IRE-TK-CAT containing the TK promoter without or with the IRE element cloned upstream (Figure 11 panel A). The HL60 cell line was chosen because, for our purpose, it was important to determine the activity of this element in a myeloid environment.

The results of the CAT assay are shown in Figure 11 panel B. In the HeLa cells transfected with the pBL-IRE-TK-CAT plasmid, IFN-γ increases transcription of the reported gene of 40 times compared to the CAT activity of the cells transfected with the same plasmid in absence of treatment. In the HL60 cell line, the enhancement upon IFN-γ treatment is about 20 times. The cells transfected with the plasmid pLB-TK-CAT did not show any increase of CAT activity upon treatment with IFN-γ, demonstrating that the effect seen with the pLB-IRE-TK-CAT plasmid is not just due to a more general effect on cellular transcription of the drug.

Given these promising results, the IRE element was cloned upstream the TK promoter in the pBabeHygro/TK/gp91-*phox* vector (Figure 9). Infectious viral preparation were produced through the use of the PA317 packaging clones as described for the other two vectors containing

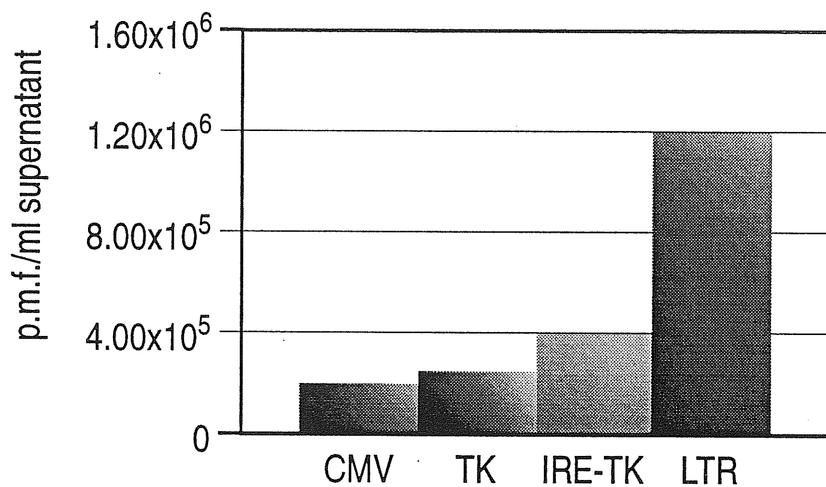
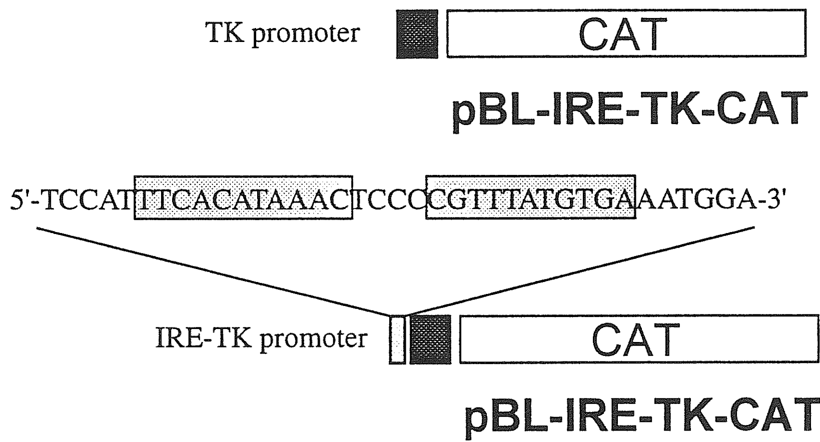


Figure 10. Retroviral titers obtained for the different retroviral vectors.

The titers for the four retroviral vectors have been analyzed by means of quantitative PCR (p.f.m.=provirus molecules formed). For each retroviral vector, the values obtained from the best producer packaging clone are shown.

LTR: pBabeHygro/gp91-*phox*; TK: pBabeHygro/TK/gp91-*phox*; CMV: pBabeHygro/CMV/gp91-phox; TK-IRE: pBabeHygro/TK-IRE/gp91-*phox*.

A



B

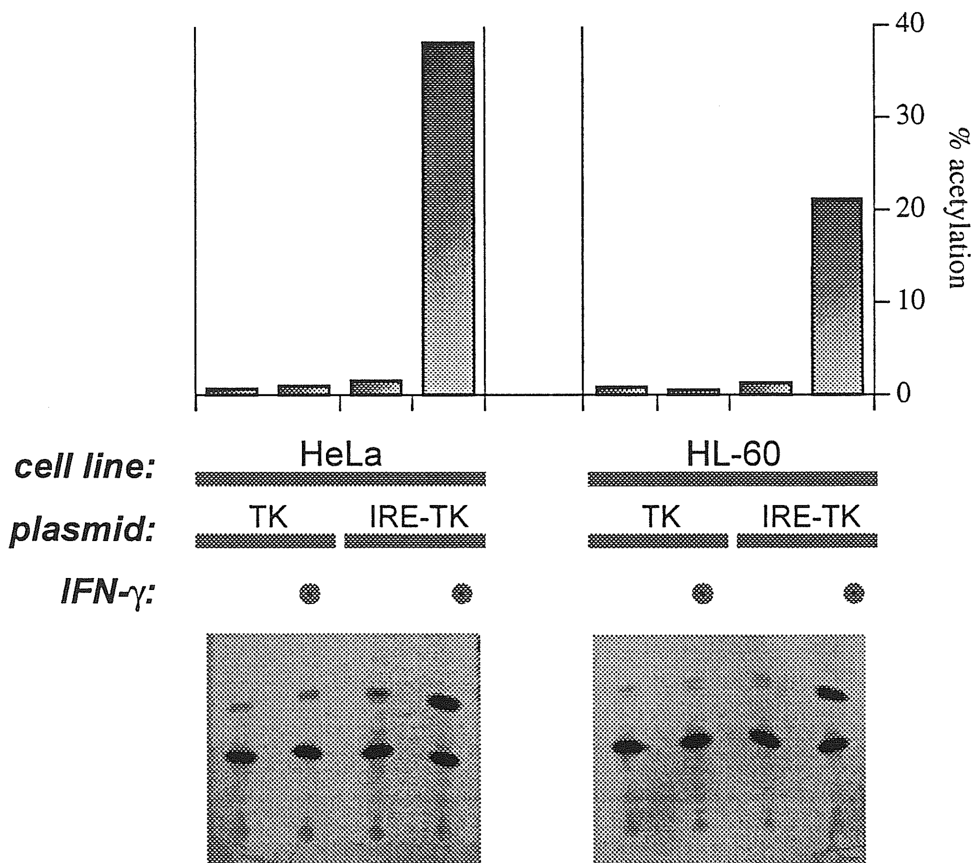


Figure 11. Activity of the IRE element in HeLa and HL60 cell lines. CAT assays were performed to detect the chloramphenicol transacetylase activity in the HeLa and HL60 cell lines. Cells were tranfected with plasmids pBL-TK-CAT and pBL-IRE-TK-CAT (panel A) and the CAT activity was determined after 24 hours of stimulation with interferon-γ. In panel (B), the results of the CAT assay are shown in both cell lines (HeLa, HL60), with both plasmids (TK, IRE-TK), with and withouth interferon-γ stimulation (IFN-γ), and without it.

These experiments have been performed at least three times obtaining consistent results; only the results of one experiment are shown.

the internal promoters. Fifty packaging clones were tested via quantitative PCR to identify the best producer. The highest titer detected for this vector was $3.5x10^5$ integrated proviral molecules/ml supernatant, about ten times lower than the titer obtained for the vector without any internal promoter (Figure 10).

2.2 Comparison of the activity of the four retroviral vectors in HeLa cells

An homogeneous system was required in order to compare the activity of the four different promoters in the context of the retroviral vector backbone. To avoid the bias of different efficiency of infection given the different titers of the viral preparations, transfection experiments were performed in HeLa cells with the four retroviral plasmids. Since selection for the hygromycin-resistance gene presence in the vector backbone could interfere with expression of the upstream promoters, the retroviral plasmids were co-transfected with plasmid pSV2neo, and resistant cells were selected with G418.

After 4 weeks of selection the cells were harvested for RNA extraction. HeLa cells transfected with the plasmid pBabeHygro/IRE-TK/gp91-*phox* were splitted in two Petri dishes and one culture was treated with IFN-γ. The extracted RNA was analyzed by Northern blotting for the presence of the gp91-*phox* transcript .

Northern blotting analysis on the four HeLa cell lines transfected with the four retroviruses, revealed that the gp91-*phox* transcript obtained from the pBabeHygro/gp91-*phox* vector was the more abundant one, while the transcription driven by the TK vector and from the IRE-TK vector resulted less efficient (Figure 12). Moreover, stimulation of the cells with IFN-γ did not result in an increase of the gp91-*phox* mRNA. No transcript were detectable in the cells transfected with the CMV vector. The transcripts transcribed from the viral LTR have a length of 5770 bp for the TK vector, 6370 bp for the CMV vector and 5470 for the vector without any internal promoter. Instead, the transcript from the TK and the CMV promoter is 4570 bp. The length of the transcripts detected showed that in the TK vectors, the transcription was driven mainly by the internal promoters. This observation could explain the reduced titer obtained with these internal promoter vectors.

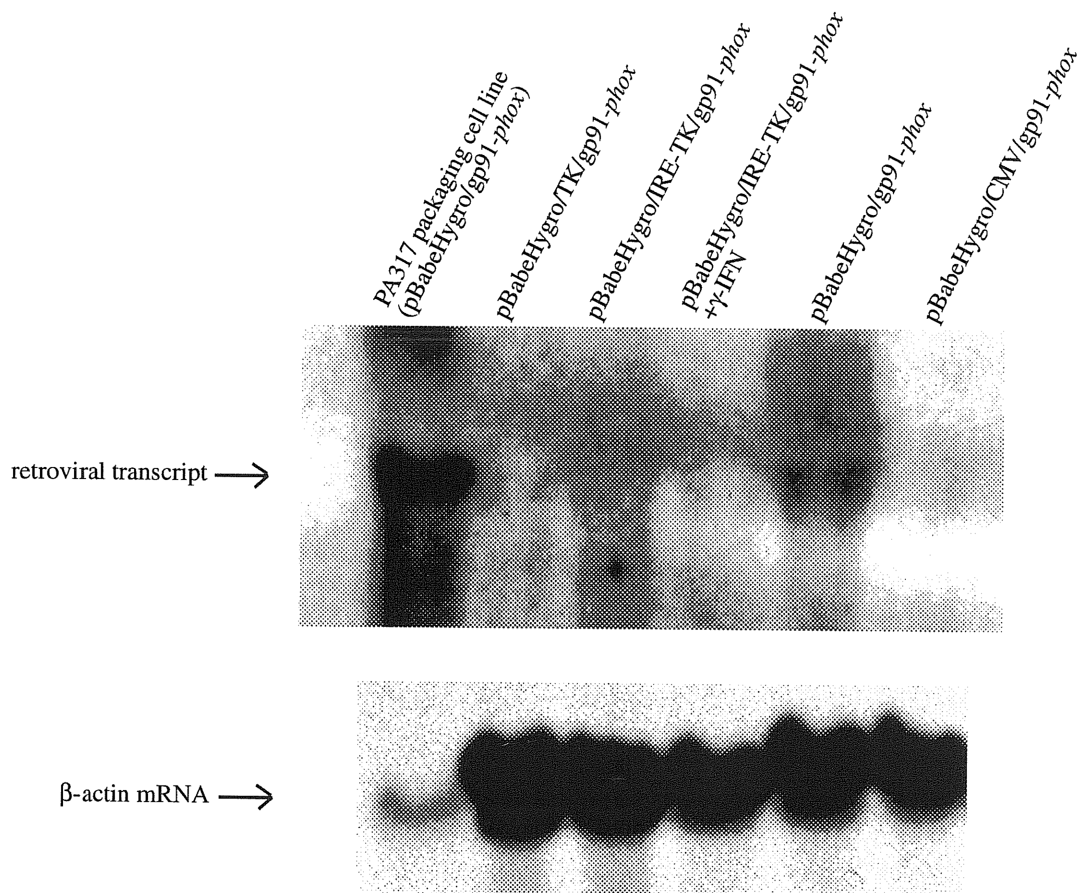


Figure 12. Northern blotting of HeLa cells tranfected with the four retroviral plasmids. Retroviral mRNA detected in HeLa cells transfected with the the retroviral plasmids pBabeHygro/gp91-*phox*, pBabeHygro/TK/gp91-*phox*, pBabeHygro/IRE-TK/gp91-*phox* and pBabeHygro/CMV/gp91-*phox*. The cells transfected with the plasmid containing the IRE element have been treated with γ-IFN 1000u/ml for 24 hours (pBabeHygro/IRE-TK/gp91-*phox* +γ-IFN). The packaging cell line PA317 producing the retroviral vector pBabeHygro/gp91-*phox* has been used as a positive control . The β-actin transcript is shown as a loading control.

3. Development of a quantitative PCR procedure for viral titering

We developed a strictly quantitative PCR procedure for the determination of the retroviral titer, based on competitive PCR. This approach consists on the simultaneous PCR amplification of the sample and of a DNA competitor fragment. This competitor shares most of its sequence with the target template (including primer recognition sites), but is slightly different in size from the latter, and hence easily distinguishable by gel electrophoresis. By this approach, the two molecular species compete for amplification, and any predictable or unpredictable variable affecting amplification has identical effects on both species. As a consequence, the final ratio between the yield of the two amplification products is identical to the initial ratio before amplification. Since the number of added competitor molecules is known, the amount of target can be easily calculated. In the last few years, we have extensively used these procedures for the exact quantitation of nucleic acids in a variety of experimental and clinical situations (Comar et al., 1995; De Rossi et al., 1996; Giacca et al., 1994; Grassi et al., 1995; Grassi et al., 1994; Menzo et al., 1992; Sestini et al., 1995; Tafuro et al., 1996).

PCR-titration by competitive PCR was obtained by measuring the number of viral RNA genomes in producer cell clones supernatants and the number of proviral DNA genomes formed after infection of NIH3T3 cells, and these results were compared with those obtained by a standard titration method based on the NIH3T3 colony assay (see flow chart in Figure 13).

An example of the developed competitive PCR procedure is presented for the determination of the titer of the retroviral vector pBabeHygro/gp91-*phox*, expressing the gp91-*phox* cDNA from the Moloney LTR (see previous section). A schematic representation of this vector is presented in Figure 14 panel A. For the PCR experiments, two primers were selected within this construct, located at the 3' end of the gp91-*phox* coding region (primer CGD17) and within the SV40 early promoter (primer SV1ter), as indicated in Figure 14 panel A. Amplification with these two primers generates a 242 bp product from the viral construct template. The competitor for competitive PCR experiments was constructed by the recombinant PCR methodology (Diviacco et al., 1992; Grassi et al., 1994) using two contiguous internal primers with a 5' tail, as shown in Figure 14 panel B.

This competitor consists of a DNA fragment corresponding to the amplification product obtained with primers CGD17 and SV1ter with the insertion of 20 additional base pairs in the middle, to allow resolution of the two species by gel electrophoresis (Figure 14 panel B). Two examples of proviral copy number determination by competitive PCR are shown in Figure 14 panel C. For each determination, a fixed amount of sample was amplified in the presence of the indicated amounts of competitor molecules. After amplification, the PCR products were resolved by gel electrophoresis, stained with ethidium bromide and quantified by densitometric scanning. According to the principles of competitive PCR, the number of competitor molecules initially added to the reaction is linearly related with the ratio between the two final amplification products. From the equation describing this relationship, the number of molecules

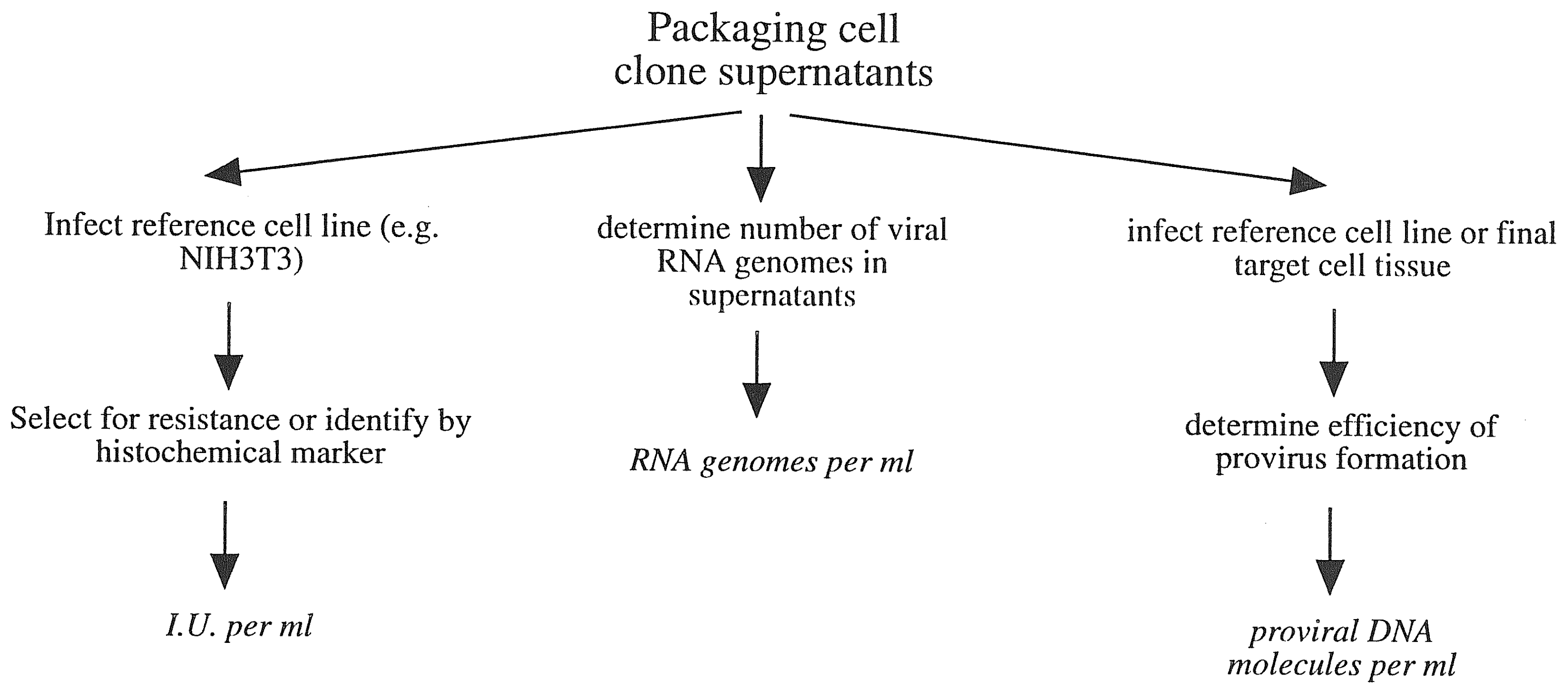


Figure 13. Flow chart for retrovirus titer determination

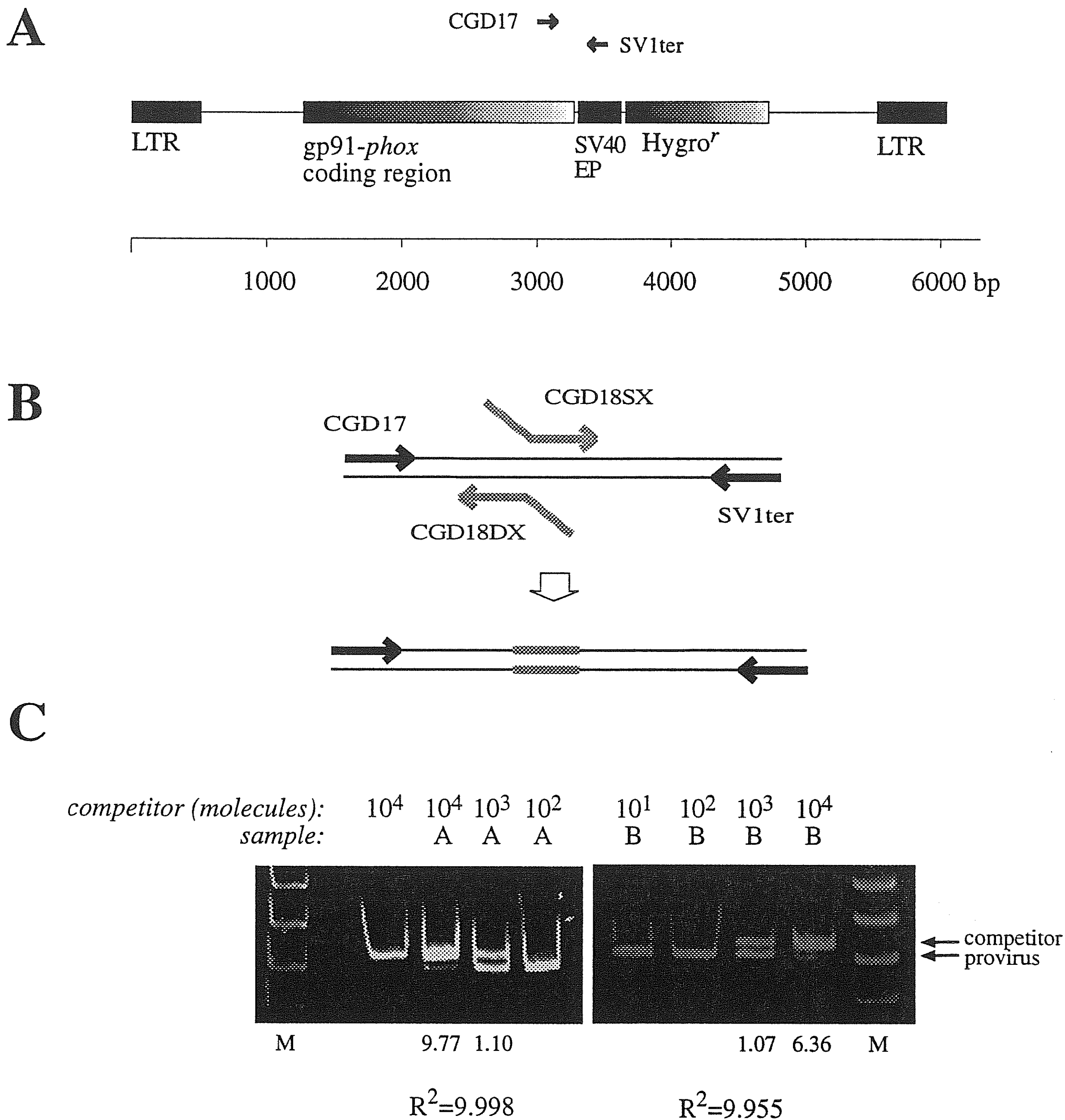


Figure 14. Competitive PCR for viral DNA and RNA quantitation

(A). Schematic representation of the pBabeHygro/gp91-*phox* retroviral construct used in this study. The location of the relevant genetic elements is indicated (LTR: long terminal repeat; SV40EP: SV40 early promoter; Hygro^r: hygromicin resistance gene). Primers used for PCR amplification are indicated by arrows. Primer SV1ter s located in the SV40 early promoter and primer CGD17 in the gp91-*phox* coding region. Amplification with these primers generates a 224 bp product from the viral construct template.

(B). Construction of the competitor DNA molecule for competitive PCR. The black arrows indicate primers CGD17 and SV1ter, used for amplification; the gray arrows contain primers CGD18SX and CGD18DX used for competitor construction.

(C). Virus DNA and RNA quantification by competitive PCR. Competitive PCR was applied to measure the concentration of viral genomes in packaging cell clones supernatants and proviral DNA formed upon infection of NIH3T3 cells.The Figure shows the results of amplification of proviral DNA in infected NIH3T3 lysates for two samples (A and B). The ratio between the intensity of the upper (competitor) and lower (provirus) - reported in the lower part of the gels - is linearly correlated with the input number of molecules, as shown by the correlation coefficient (lower part of the Figure). M: molecular weight marker.

corresponding to a 1:1 ratio is evaluated. This value corresponds to the number of target viral molecules present in the reaction.

3.1 Comparison of different titration procedures

Three titration procedures were compared using the supernatant of several virus-producer cell clones obtained by a two-step packaging cell line passage with pBabeHygro/gp91-*phox*: direct quantification of virion RNAs in the cell clone supernatants; quantification of proviral DNA molecules formed 24 hours after infection of NIH3T3 cells; quantification of colonies obtained by hygromycin selection of infected NIH3T3 cells (Figure 13). A correlation analysis was performed between titers measured by quantitative RT-PCR on the supernatants of packaging cell lines (number or RNA genomes/ml supernatant) and titers measured by the colony assay (number of hygromicin-resistant colonies obtained/ml) for 32 independent clones (R^2: 0.32). The same analysis was performed with the values (provirus molecules formed/ ml supernatant) obtained by competitive PCR from the DNA extracted from the cells at 24 hours after infection with the viral supernatant for 20 independent clones (R^2: 0.97).

The results of these analyses are presented in the graphs of Figure 15, where the colony assay results are plotted against the number of virion RNA molecules or the number of proviral DNA molecules (A and B respectively). As shown in panel A, the number of viral RNA genomes in the producer cell clone supernatants is a poor predictor of the efficiency of infection. In simpler terms, the data obtained show that the same efficiency of stable infection (as evaluated by the number of NIH3T3 selected colonies) can be obtained by inocula containing an extremely variable number of viral genomes. The main determinant in the discrepancy between the two measurements is likely to be due to the presence of non-infectious, defective particles in the producer cell supernatant. Using a DNA-competitor, on the contrary, the determination of proviral DNA copy number at 24 hours after infection of NIH3T3 cells is linearly correlated with the results of the colony assay (correlation coefficient: 0.97; panel B). These data indicate that, while the determination of the levels of viral RNA genomes in the supernatant gives an unreliable estimate of the efficiency of infection, the measurements of formed provirus DNA is a good predictor for titer determination.

In order to exclude any possible artifact in the competitive PCR procedure for proviral DNA determination, the supernatant from a high producer clone was inactivated for one hour at 55°C prior to the infection of the NIH3T3 fibroblasts. This experiment confirmed the specificity of the technique as neither viral DNA was detected by PCR nor resistant colonies were obtained by the colony assay.

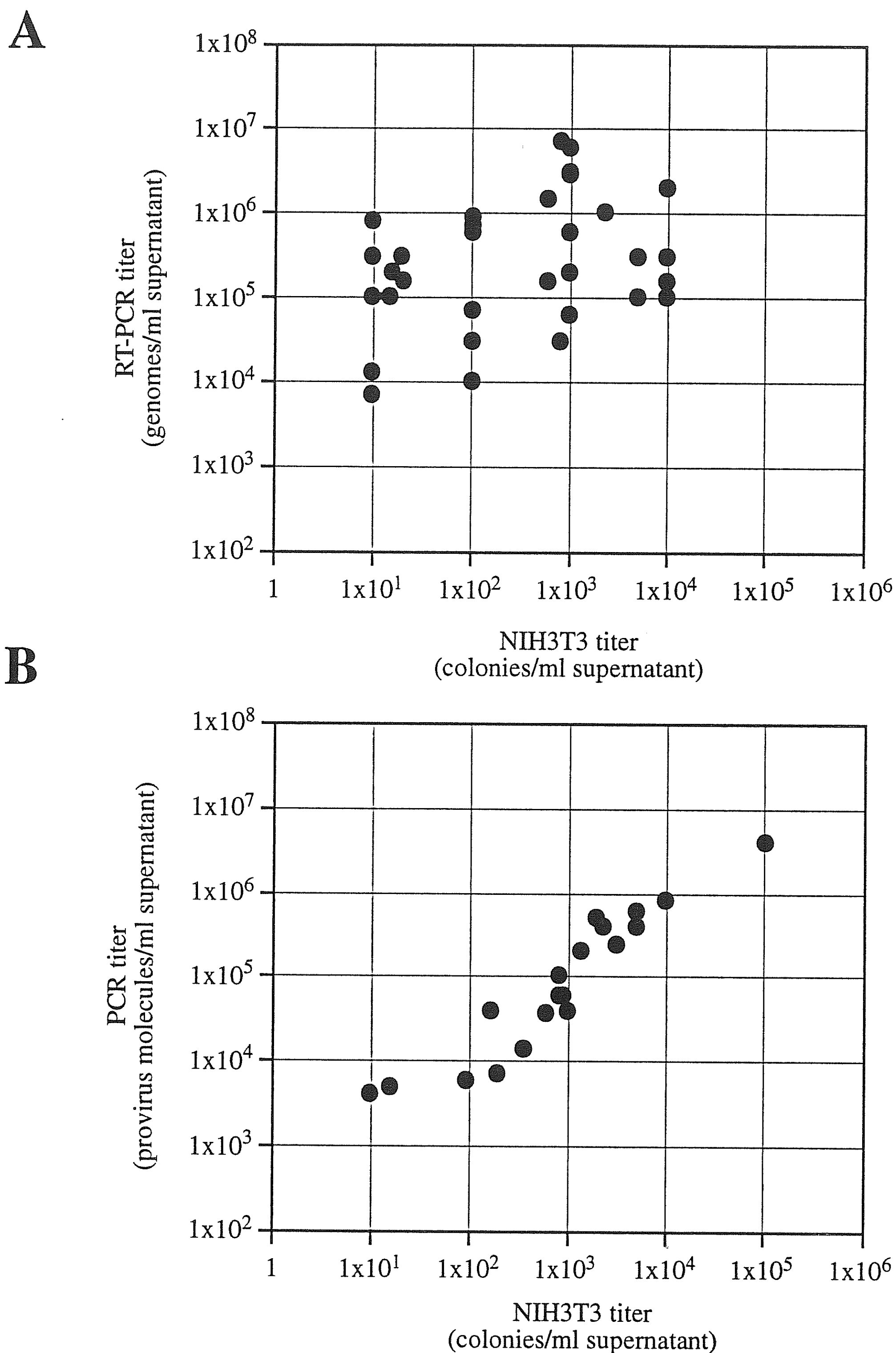


Figure 15. Correlation analysis between titers obtained by competitive PCR and titers obtained by the NIH3T3 colony assay.
(A) Correlation between titers measured by quantitative RT-PCR on the supernatants of packaging cell lines (number of RNA genomes per ml) and titers measured by the colony assay (number of hygromicin-resistant colonies obtained per ml) for 32 indipendent clones (r2: 0.32).
(B) Correlation between titers measured by quantitative PCR on NIH3T3 lysates (number of provirus DNA molecules formed (p.m.f.) at 24 h after infection) and titers measured by the NIH3T3 colony assay for 20 independent clones (r2: 0.97).

3.2 Stability of proviral DNA copy number at different times after infection

The analysis of the data presented in Figure 13 revealed that the number of provirus molecules formed at 24 hours post infection of NIH3T3 cells is from 40 to 400 times (mean value: 128 times, standard deviation: 103.8) higher than the actual number of colonies obtained after a two-week selection. A possible explanation for this observation is that most of the reverse-transcribed products formed upon infection are lost during long term culture. It has been already reported for the avian sarcoma virus and for other viral systems that in acutely infected cells the unintegrated DNA represents more than 75% of the viral DNA (Varmus et al., 1976). To further explore this possibility, a time course experiment was carried out on NIH3T3 cells in order to measure the proviral copy number at different times after infection. Cells were transduced using the supernatant of a packaging cell clone (clone 36), and a fixed number of cells were harvested at days 1, 7, 14, and 21 after infection. The competitive PCR procedure was performed on the cellular lysates in order to measure the number of proviral DNA molecules present at the different time points. The results of this experiment, which are shown in Figure 16 panel A, clearly indicate that the number of proviral molecules per number of cells remains grossly unchanged at the different time points. This observation was further reinforced by the analysis of proviral formation in two different human cell lines (HeLa, 293) upon infection with the same retroviral clone (Figure 16 panels B and C respectively), and upon infection of NIH3T3, HeLa and 293 cells with another retroviral clone (clone 52, Figure 16 panels E to G respectively). The results obtained show that the number of proviral DNA molecules remains almost unaltered during the first two weeks after infection.

These observations clearly indicate that the actual discrepancy between the standard titer measured by NIH3T3 selection and the PCR titer evaluated from the number of proviral DNA molecules is not due to the lost of unintegrated proviral DNA molecules upon long term culture. Interestingly, while the proviral PCR titers for each of the analyzed clones (shown in Figure 16 panels A-C and E-G) were very similar for the different cell lines, the respective titers measured by hygromycin selection were different. The colony assay titer obtained after selection of NIH3T3 cells was ~10 times higher than the respective titer measured on HeLa cells and ~20 times higher than the one evaluated by infection of 293 cells. These results are shown in Figure 17 panels A and B for the two analyzed viral clones, and are expressed as a ratio between the PCR titers and the hygromicin resistance titers. Since the rate of infection is roughly the same for all the three cell lines (as demonstrated by the PCR quantifications), the differences detected in the number of colonies after selection are probably a consequence of the different rates of expression of the hygromycin resistance gene in the different cellular contexts, or of the different efficiency of colony formation by each cell line. Both possibilities, however, are not related to the actual efficiency of infection of the viral preparations. In particular, it can be assumed that the titers calculated by the colony assays for the retroviral construct used in this study underestimate its actual efficiency of infection, because of the poor expression of the

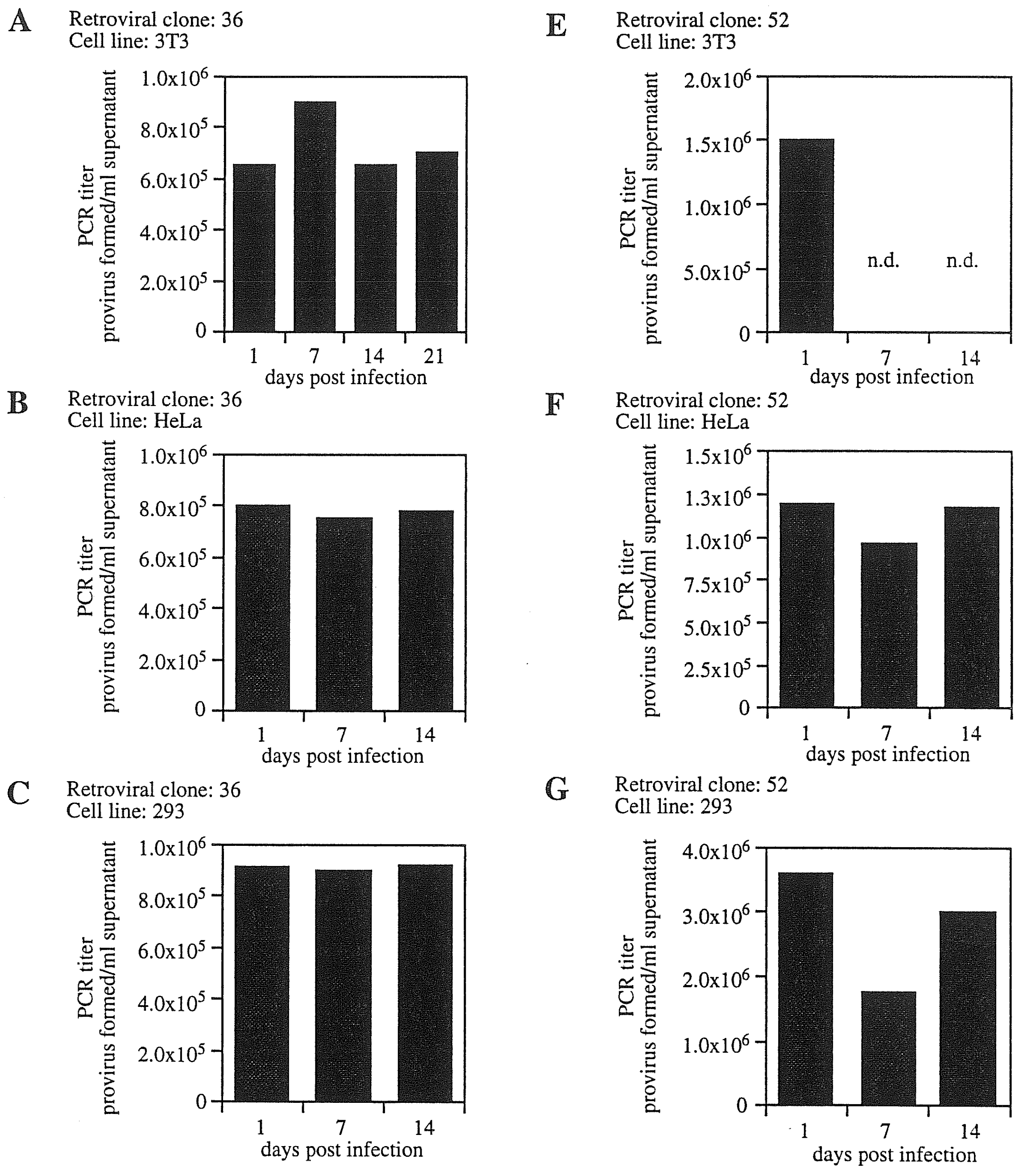


Figure 16. Provirus copy number determination at different times upon transduction of NIH3T3, HeLa and 293 cells.

A time course experiment was performed by transduction of 2x105 murine NIH3T3, 2x106 human HeLa and 1.5x106 human 293 recipient cells, seeded on 60 mm dishes. Supernatants from two different producer clones (clones 36 and 52 of pBabeHygro/gp91-phox) were used for transduction. At 24 hours after infections, cells were seeded on 10 mm dishes and then harvested at days 1, 7, 14 post infection (HeLa and 293) or days 1, 7, 14 and 21 (NIH3T3) for provirus copy number determination by competitive PCR.

For the colony assays, cells were selected in the presence of 150 U/ml (HeLa and NIH3T3) or 250 U/ml (293) of hygromicin B. Titers were estimated from the number of colonies after 12 days of selection.

Both PCR and colony assay titers are expressed per ml of packaging cell clone supernatant.

The results represt the mean values of at least two independent experiments.

Panels A, B and C. Titers measured by competitive PCR on lysates from NIH3T3, HeLa and 293 cells respectively, at the indicated times after transduction with the supernatant from the packaging cell clone #36.

Panels E, F and G. Same as panels A-C, by using the supernatant from the packaging cell clone #52. n.d. = not done

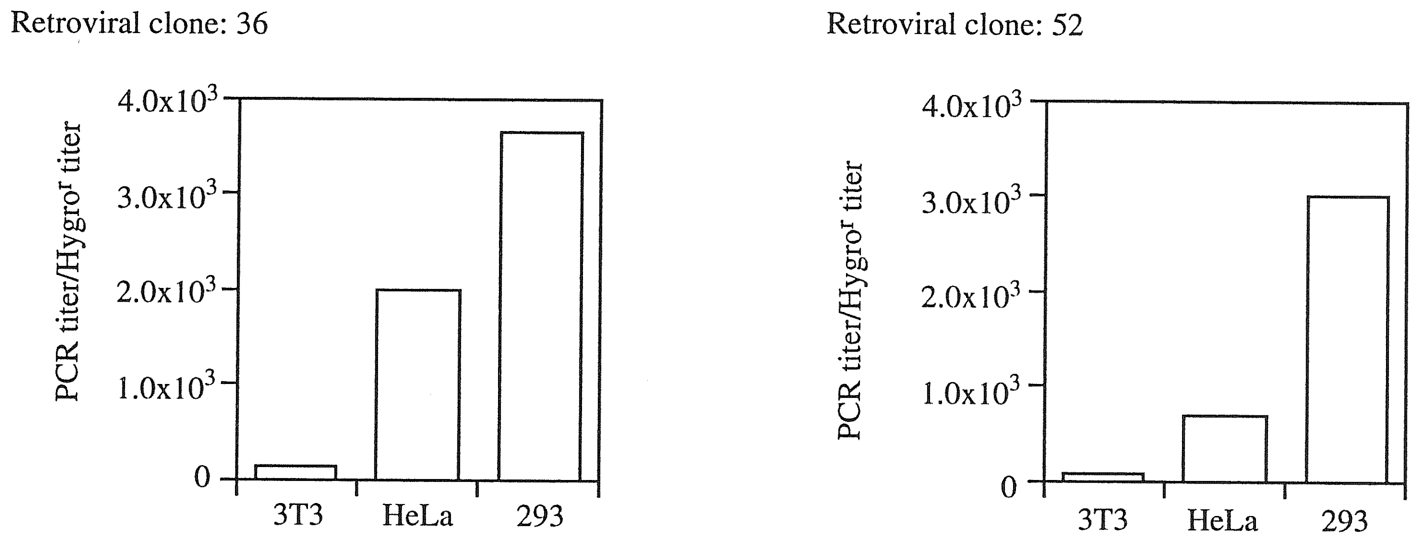


Figure 17. Ratio between the PCR titers at day 1 and the hygromicin-resistance titers for the NIH3T3, HeLa and 293 cell lines. The results represent the mean values of at least two indipendent experiments.
(A) Infection with the producer cell clone supernatant #32.
(B) Infection with the producer cell clone supernatant #52.

hygromicin resistance gene from the SV40 promoter. This event is likely to occur in retroviral vector backbones containing two promoters. (Miller et al., 1990; Nakajima et al., 1993)

Section C

4. TRANSDUCTION OF GP91-*PHOX* IN PBL 985 X-CGD CELLS, B-CELL LINES AND IN HEMATOPOIETIC PROGENITORS

4.1 Functional correction of PLB-985 X-CGD cells

The promyelocytic cell line PLB-985 X-CGD is an in vitro model representing a myelocytic cell line in which the gp91-*phox* gene has been disrupted (Zhen et al., 1993). For this reason, it offers the important opportunity to compare the efficacy of different retroviral vectors in a specific myeloid environment.

PLB-985 X-CGD cells were infected with the four retroviral vectors through coculture with the packaging cell lines for two days. Competitive PCR analysis demonstrated that the percentage of infection varied around 10% for all the four vectors. After one month of hygromycin selection, the cells were differentiated adding DMF (dimethylformaldehyde) at a final concentration of 0.5% for 5 days and analyzed by the luminol assay to detect the production of superoxide. The results obtained are shown in figure 18. The functional reconstitution of the cells, as detected from the oxidation of luminol, varied in two different experiments from 5.6 to 8% of the activity of the normal control, represented by the original PLB-985 cell line. No substantial differences in the level of reconstitution could be detected for the four different retroviral vectors. The stimulation for 24 hours with IFN-γ did not increase the functional activity of the gp91-*phox* gene driven by the IRE-TK promoter.

4.2 Transduction of X-CGD B-cell lines

The pBabeHygro/gp91-*phox*:vector (driving expression of the transgene from the LTR promoter) was also used for transduction of EBV-transformed B lymphoblastoid cell lines established from two X-CGD patients (A.Z. and A.G). B-cells were infected by coculture with the best packaging cell clone for two days in presence of polybrene. Cells were then selected for several weeks in hygromycin-containing medium. Several hygromycin resistant clones were isolated and kept in culture for several weeks.

The transduced clones were investigated for the presence and expression of the gp91-*phox* vector. First of all, retroviral vector integration in the hygromycin resistant clones was analyzed. Total genomic DNA was digested with *Kpn* I or with *Xba* I, both of which cut once in the proviral LTRs, and hybridized with gp91-*phox*-specific probe. Figure 19 panel A shows that a proviral band of the expected size with no apparent rearrangements is present in each one

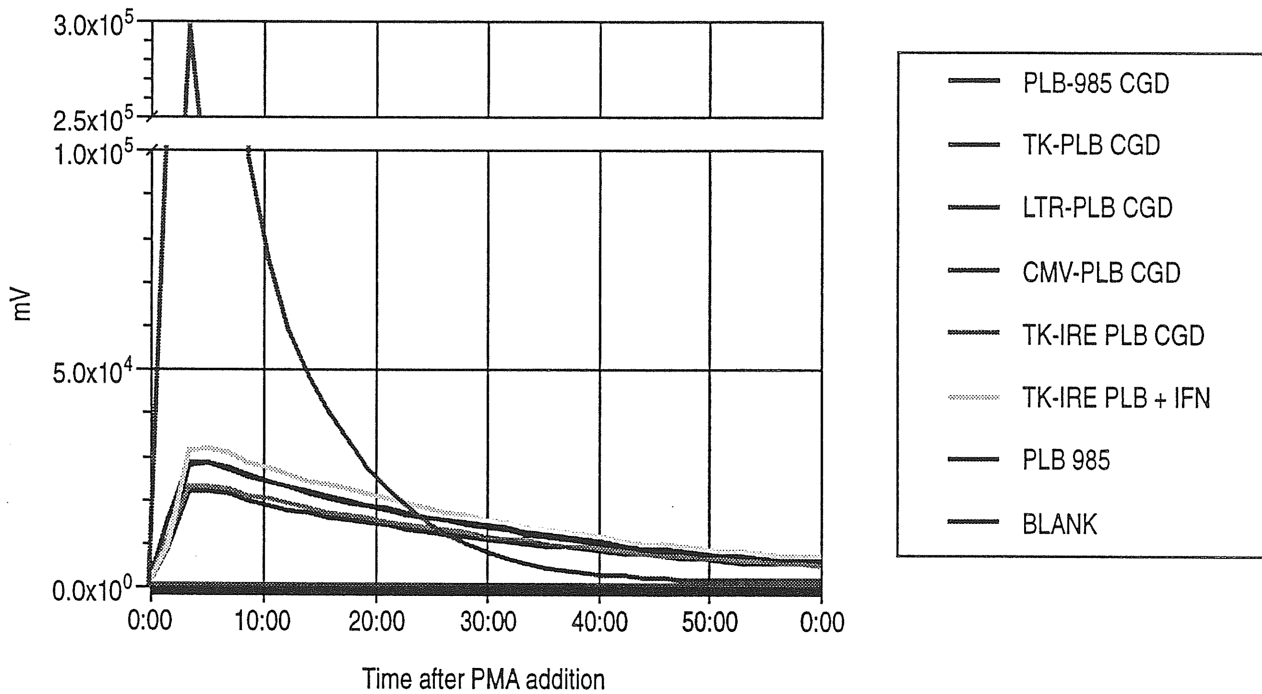
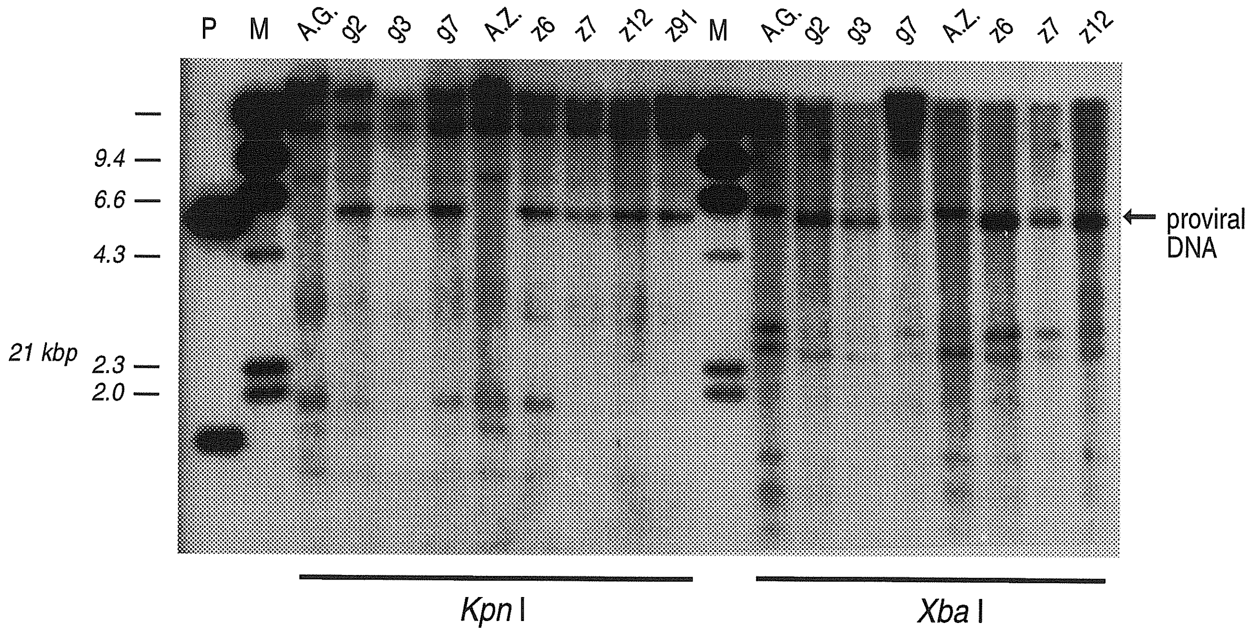


Figure 18. Superoxide production by the PLB-985 X-CGD cell line infected with the four retroviral vectors (luminol assay).
PLB-985 X-CGD cells were infected with the retroviral vectors pBabeHygro/gp91-*phox*, pBabeHygro/TK/gp91-*phox*, pBabeHygro/CMV/gp91-*phox* and pBabeHygro/IRE-TK/gp91-*phox* and assayed for superoxide production after differentiation. The cells infected with the pBabeHygro/IRE-TK/gp91-*phox* vector were stimulated with 1000 U/ml of interferon-γ prior to the assay.

A

Southern blotting



B **Northern blotting**

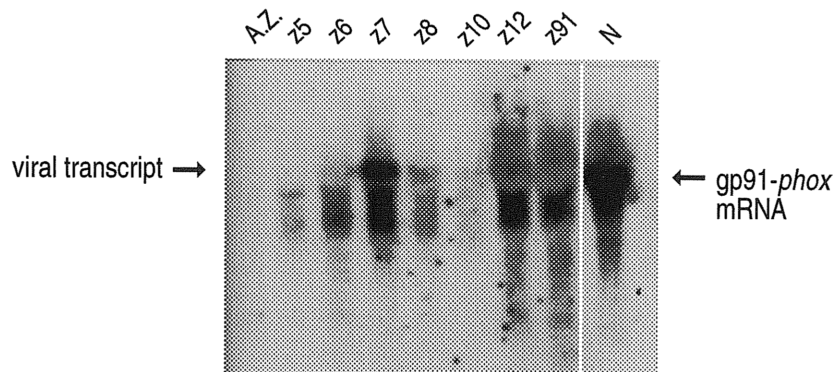


Figure 19. Analysis of transduced B-cell clones.
(A) Southern blotting.
DNA extracted from transduced lymphoblastoid B cell clones from patient A.G. (clones g2, g3, g7)) and A.Z. (z6, z7, z12, z91) was digested with Kpn I or Xba I, both of which cut once in each viral LTR, and probed with a labeled gp91-*phox* cDNA fragment. The arrow indicates the proviral DNA band. A.G. and A.Z.: DNA from nontransduced cell lines; M: labeled molecular weight marker; P: plasmid pBabeHygro/gp91-*phox* digested with Kpn I.
(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 b-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 nontransduced parental B cell line.

of the analyzed clones. Analogous results were obtained using the hygromycin specific probe (data not shown). From the intensity of the hybridization signals obtained, it can be estimated that the proviral construct is present in no more than one or two copies per cellular genome, considering as a reference the signal corresponding to the endogenous gene.

Expression of the transduced construct was tested by Northern blotting and hybridization with the gp91-*phox* cDNA probe (see Figure 19 panel B for clones derived from patients A.Z., in which expression of the endogenous mutated gene is very low). The results obtained indicate that the LTR-driven expression of the gp91-*phox* transcript is highly variable from clone to clone, probably reflecting the effects of different retrovirus integration events. Interestingly, expression of the gene in some clones (see, for example, clone z7 in Figure 19 panel B lane 4) is almost as high as that detectable in control B-lymphoblasts from normal individuals (lane 9).

To verify that the gp91-*phox* protein was produced and correctly folded, some of the transduced B-cell clones from patient A.Z. were investigated by Western blotting using the MOAB48 (Verhoeven et al., 1989). In these experiments, the protein typically appears as a smear of products with a relative mass from 70 to 90 kDa, due to the high extent of glycosylation of the protein core (Dinauer et al., 1987). To obtain a stronger and homogeneous signal on the immunoblot, the membrane fraction preparations were treated with endoglycosidase F that removes the glycosyl residues and reveals the 55 kDa core protein. As shown in Figure 20, while the gp91-*phox* protein is undetectable in the untransduced parental cells of the patient, it could be revealed in the two transduced clones analyzed. These clones are the ones which also show high levels of expression of gp91-*phox* mRNA.

To assay for the restoration of the oxidase enzymatic function in the transduced cell lines, the highly sensitive luminol assay was used. While the lymphoblastoid cell lines from the patients were not able to produce any detectable amount of H_2O_2 upon PMA stimulation, a net increase in activity was detected in the hygromycin resistant clones from patients A.G. and A.Z. obtained after infection with pBabeHygro/gp91-*phox* (Figure 21 panels A and B respectively). In these clones, a wide variability in the amount of superoxide generated could be observed, probably reflecting the detected variability in the efficiency of gp91-*phox* gene expression. Despite this variability, it should be observed that the values of oxidase activity obtained for most of the clones fall in the same range as those obtained from some normal cell lines.

4.3 Transduction of hematopoietic progenitors

Considering that the final target cell for the correction of the defects leading to CGD is the hematopoietic pluripotent stem cell, the vectors were used to transduce hematopoietic precursors derived from peripheral blood of normal donors and CGD patients.

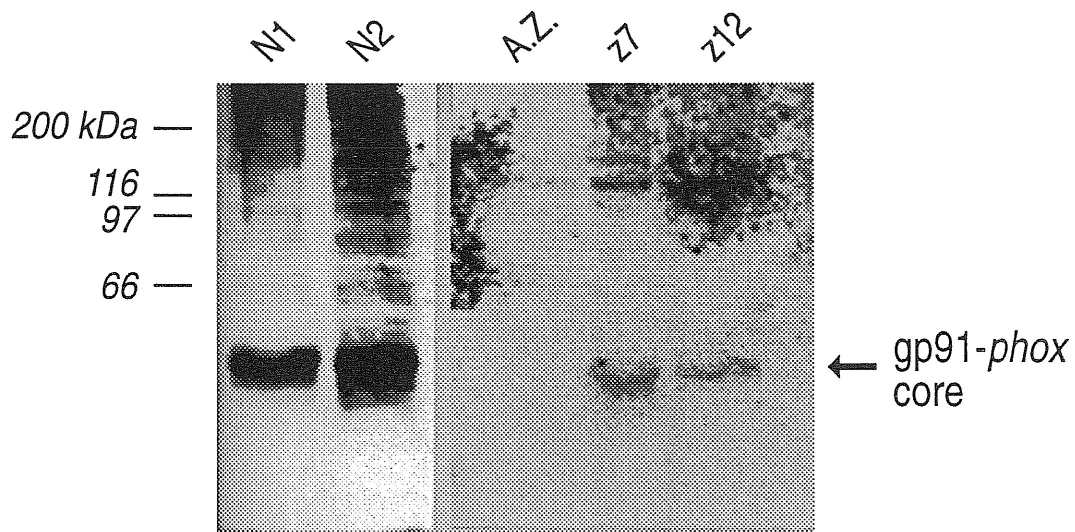
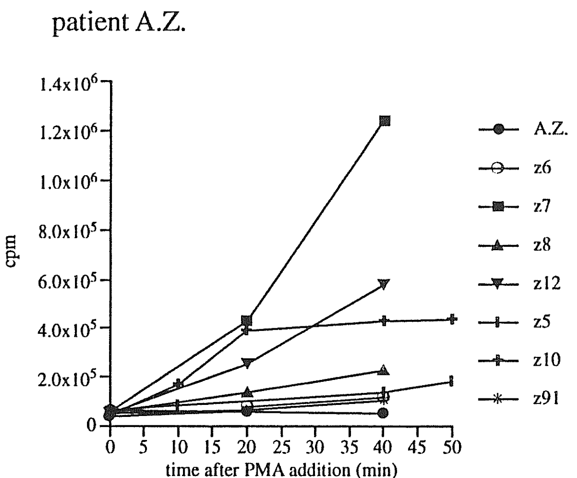


Figure 20. Western blotting of transduced cell clones.
Cellular membrane fractions obtained from lymphoblastoid cell lines derived from normal individuals (N1 and N2), from patient A.Z., and from transduced clones derived from patient A.Z. (z7 and z12) were digested with PNGase F to obtain the core polypeptides, resolved by SDS-PAGE, blotted, and probed with the monoclonal antibody MoAb 48 against gp91-*phox*.

A



B

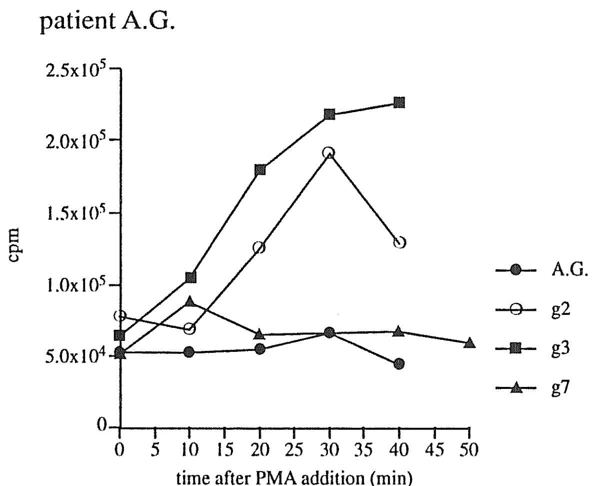


Figure 21. Functional correction of NADPH oxidase activity in transduced B-cell clones. The graphs represent the production of O_2^- as evaluated by a chemiluminescent luminol assay in parental lymphoblastoid cell lines of patients and transduced cell clones (patient A.Z. in panel A, patient A.G. in panel B).

Conditions were established to optimize the in vitro proliferation and retroviral transduction of hematopoietic precursors. In our experience and according to published data, the addition of a cytokine cocktail composed of Il-3, Il-6, GM-CSF, SCF to the progenitor cell culture produces the most satisfactory results (Dick et al., 1991; Nolta et al., 1990). Transduced and control, nontransduced precursors were cultured in methylcellulose semisolid medium for 14 to 20 days. This assay provides evidence for the presence of progenitor cells that are able to produce myelomonocytic and/or erythroid cell lineages according to the growth factors added to the culture. No bias toward any cell lineage or general reduction of the clonogenic efficiency that could be related to the infection with retroviruses could be detected.

The single colonies were analyzed by means of PCR to detect the presence of proviral DNA. The results of two separate experiments are shown in Table IX. The percentage of transduction varies between 100% to 80% for the vector without any internal promoter and from 30% to 40% for the vectors containing the internal promoters. Given the poor outcome of the infection of PLB 985 X-CGD cells with the vector containing the IFN-responsive element, this vector was not used.

Analysis of the O_2^- production of myeloid colonies after 21 days of culture in semisolid medium using the NBT assay revealed reconstitution of the activity of the NADPH oxidase complex, detected from the intensity of staining (Figure 22). The comparison between the colonies infected with the three vectors pBabeHygro/gp91-*phox*, pBabeHygro/TK/gp91-*phox* and pBabeHygro/CMV/gp91-*phox* did not reveal a strong difference in the intensity of staining (it should be considered, however, that the NBT test cannot be considered a quantitative assay). These data show that the presence of two internal promoter do not markedly interfere with the transcription in the hematopoietic environment.

4.4 Quantitative assessment of transduction of hematopoietic precursors

Further transduction experiments were carried out with the BabeHygro/gp91-*phox* vector. Transduction experiments were carried out with six different cellular samples, using peripheral blood or bone marrow mononuclear cells from normal and X-CGD individuals, either without or after enrichment for $CD34^+$ progenitors, as indicated in Table X. Purification of $CD34^+$ cells was performed using a commercial anti-CD34 monoclonal antibody affinity column. This procedure gives variable results using individual cell samples. In our experience, the enrichment in clonogenic precursors with respect to unselected mononuclear cell preparations is in the range of 10-35 fold (data not shown).

The number of the different colonies obtained from transduced and non transduced hematopoietic precursors was compared and no difference was scored in the type and number of hematopoietic colonies, demonstrating that the viral infection does not influence the differentiation of the precursors (data not shown).

experiment #	pBabeHygro/ gp91-*phox*	pBabeHygro/TK/ gp91-*phox*	pBabeHygro/CMV/ gp91-*phox*
1	100%	30%	40%
2	90%	40%	30%

Table IX. Percentage of retrovirus-positive myeloid colonies upon infection with the indicated retroviruses in two independent experiments

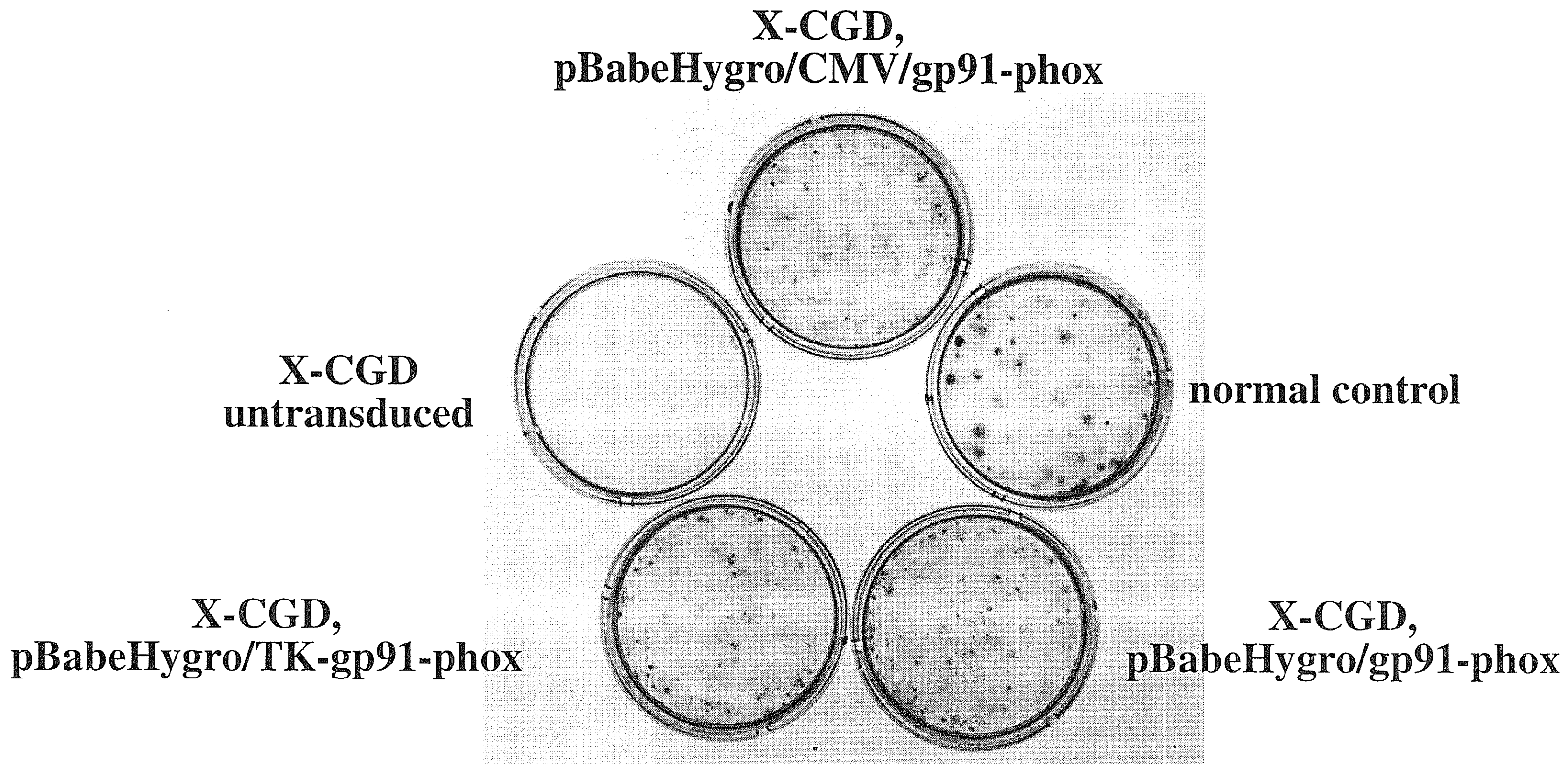


Figure 22. Functional reconstitution of NADPH activity in hematopoietic precursors with the retroviral vectors pBabeHygro/gp91-phox, pBabeHygro/TK/gp91-phox, pBabeHygro/CMV/gp91-phox.
Nitroblue tetrazolium assay (NBT) on in vitro differentiated myeloid colonies from peripheral blood hematopoietic precursors of a normal individual and an X-CGD patient. X-CGD precursors were transduced with the three different retroviral supernatants after 24 hrs stimulation with cytokines (IL-3, IL-6, SCF, GM-CSF). The intensity of the blue staining shows that all the three vectors are able to functionally reconstitute the oxidase activity in the X-CGD myeloid colonies.

Differentiated myeloid colonies from a healthy individual and untransduced colonies from the X-CGD patient are shown as positive and negative control respectively.

Efficiency of retroviral transduction

Colonies derived from transduced cells were analyzed for proviral integration in order to assess the efficiency of transduction. For this purpose, PCR experiments were performed on DNA extracted from isolated colonies with primers designed to allow the selective detection of the proviral genome. Table X shows that the number of PCR-positive colonies ranged between 100 to 60% in the different experiments. In any case, no specific amplification products were detected in colonies derived from nontransduced cells. It is interesting to observe that this high efficiency of transduction was reproducibly obtained (for both unfractionated and $CD34^+$ cells) also in the absence of any stromal support. In order to exclude that this high proportion of PCR-positive colonies could derive from artifactual cross-contamination of cells from one colony to the other, given the high sensitivity of PCR amplification, we have checked the actual number of proviral DNA copies present in 10 PCR-positive colonies (approximately composed by more than 200 cells), obtained from an additional transduction experiment. This measurement was performed by taking advantage of the competitive PCR technique describe above. DNA from these colonies was mixed with known quantities of the competitor DNA plasmid containing the same sequence amplified by the primer pair, with the exception of an inserted 20 bp sequence. An example of these amplification for two colonies is shown in Figure 23. A fixed amount of sample DNA (1/25 of total) was amplified with 20 and 200 molecules of competitor. The input copy number of target molecules in the samples was then calculated from the ratio between the amplification products obtained. In all cases, the estimated number of provirus DNA copies was roughly correlated to the size of the colony, and never below the threshold of 200 copies/colony. These quantitative data support the results obtained by qualitative PCR analysis, and clearly indicate that a high efficiency of transduction of hematopoietic progenitors was effectively obtained with this vector.

Ten out of ten erythroid colonies obtained in experiment 1 scored positive when analyzed for the presence of proviral DNA, thus ensuring that viral transduction did not interfere with the normal differentiation program of the erythroid lineage.

Analysis of proviral gp91-phox expression and function

Analysis of provirus expression was performed by RT-PCR on RNA isolated from individual colonies. Despite the high efficiency of infection achieved, only 17-20% of colonies showed expression of the proviral gp91-*phox* transcript when analyzed by RT-PCR (Table X). A greater number of colonies scored positive (50%-70%) when the RT-PCR products were further amplified for 40 cycles in a semi-nested PCR amplification (carried out for samples obtained in experiments 2, 3 and 5; see Table X). This result indicates that expression of the transduced gene occurs in the majority of infected cells, albeit at a low efficiency.

Table X. Efficiency of transduction of gp91-*phox* in hematopoietic progenitor cells

Exp	Individual	Phenotype	Cell source	CD34+ purification	Viral transduction	gp91-*phox* expression		Functional correction	
					colonies positive by PCR	*colonies positive by RT-PCR*	*colonies positive by RT-nested PCR*	*colonies positive at the NBT test*	*Average intensity of staining*
1	N1	normal	blood	-	30/30 (100%)	5/30 (17%)	n.d.	n.d.	n.d.
2	N2	normal	blood	+	5/8 (62.5%)	n.d.	5/10 (50%)	n.d.	n.d.
3	A.Z.	X-CGD	blood	-	18/19 (95%)	2/10 (20%)	7/10 (70%)	20%	+-
4	N3	normal	marrow	+	n.d.	n.d.	n.d.	95%	+++
5	V.	X-CGD	marrow	+	9/10 (90%)	4/10 (40%)	5/10 (50%)	5%	+-
6	V.	X-CGD	marrow	-	6/10 (60%)	3/5 (60%)	n.d.	20%	++

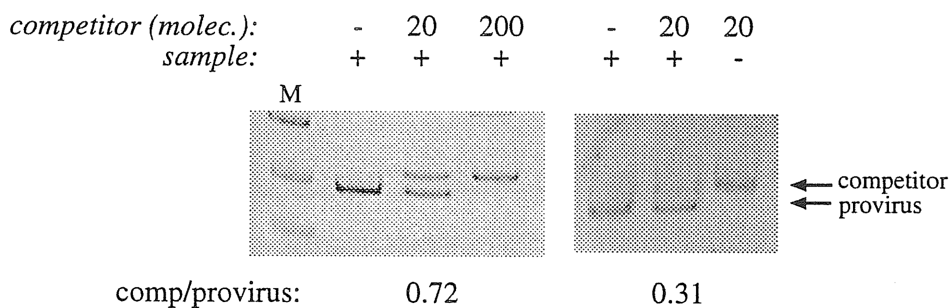


Figure 23. Competitive PCR to evaluate the number of proviral DNA copies in transduced myeloid colonies.

PCR analysis was performed with primers CGD17 and SV1ter, that amplify from pBabeHygro/gp91-*phox* proviral DNA. A fixed amount of sample DNA (1/25 of total) obtained from two transduced myeloid colonies was amplified together with the indicated amounts of a competitor DNA plasmid. The amplification products were resolved by gel electrophoresis, stained with ethidium bromide and quantified by densitometry. The bands corresponding to the competitor and provirus fragments are indicated by arrows. On the lower part of the two gels, the ratio between the two amplification products (comp/provirus) is reported. According to these estimates, the total DNA preparations from the two colonies analyzed by the gels on the left and right sides contain 694 and 1612 proviral DNA molecules respectively.

The functional correction of the oxidative defect was assessed on differentiated colonies derived from transduced cells of two X-CGD patients (experiments 3, 5, 6) by a modification of the NBT test. For each experiment, normal controls were included, as well as nontransduced X-CGD cells subjected to the same culture conditions as infected cells. The number of stained colonies were counted under an inverted microscope, and the intensity of staining was independently evaluated by two experimenters, by assigning arbitrary values ranging from - to +++.

The vast majority of mature myeloid colonies from normal individuals showed dark blue staining (+++) at the assay (see Figure 24 panel A). On the contrary, colonies from untreated hematopoietic progenitors from X-CGD patients were completely unstained (Figure 24 panel B). An average of 20% of mature colonies derived from transduced CGD precursors showed a variable but generally pale blue staining, reflecting a low level of functional correction, in agreement with the results of the RT-PCR analysis. Few corrected colonies demonstrated strong reducing activity (Figure 24 panels C and D).

Discussion

1. Characterization of the genetic defect in three patients affected by X-linked CGD

We investigated the mutations that cause X-CGD disease in three italian patients. The sequence analysis of the gp91-*phox* cDNA revealed three different point mutations in the three patients that had not been described before. This is not surprising considering the heterogeneity of mutations found in this gene. In fact the mutations are widespread in the whole coding region, with the absence of preferential hot spots. Given the very heterogeneous nature of the mutations in X-linked CGD, the precise identification of the genetic defect in a family is absolutely required for the purpose of family screening, carrier identification, prenatal diagnosis and genetic counseling.

In all the three patients analyzed, the mutations lead to the absence of the protein, probably by altering the stability of the protein and in one case the stability of the mRNA. In patient A.Z., the deletion of a the A at position 1330 leads to a premature stop codon and the loss of 100 aminoacids near its C terminus. This relatively hydrophobic region comprises the NADPH binding region and the domain involved in the binding of the cytosolic components during the activation of the oxidase (Roos et al., 1996; Rotrosen et al., 1990). 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. The mutations at the 3' splice site are less frequent than the mutations at the 5' splice sites representing just 1% of this kind of mutations (Roos et al., 1996). Splice site mutations are a common cause of disease in X-CGD patients (de Boer et al., 1992). Due to this deletion, the first nucleotide of exon 11 becomes a T, thus creating a splice acceptor sequence less favorably recognized by the splicesome (Shapiro and Senapathy, 1987). Therefore, the considerable reduction in mRNA level is possibly a consequence of suboptimal terminal splicing of the transcript with consequent mRNA instability (de Boer et al., 1992). In this regard it is worthwhile saying that we have not been able to detect any abnormally spliced mRNA species even by PCR amplification.

In the patients A.G. and N.B. the mRNA encoding the gp91-*phox* protein is present in amount comparable to the amount revealed in healthy controls and the length of the messenger RNA is correct. Besides this, the protein is absent leading to a X91^{o} phenotype also in these two cases. The detected mutations predict the occurrence of a frameshift and the generation of a downstream stop codon. In consequence, this defect generates a truncated and therefore inactive gp91-*phox* protein in both cases. In the patient A.G., the stop codon occurs at aminoacid 133 leading to the translation of just a portion of the N-terminal domain. In the patient N.B. the protein is truncated at the aminoacidic position 21.

The analysis of the nucleotide sequence surrounding the sites of the three mutations in the three patients revealed the presence of elements of interest such as short palindromes and repeated sequences. In patient A.Z., the deletion of the A in position 1330 occurs within a short palindrome AGATCT (in the sequence the deleted nucleotide is underlined) that overlaps the directly repeated sequence TCTACT (*TGAAGC*TCAAAAAG**ATCTACT/TCTACT**, with the direct repeat typed in bold letters). The deleted G at position 55 in patient N.B. is positioned within the sequence *TGAGG*GGCTCTCCATTT**TTGT**CA**TTGT**A, showing a repetition of the sequence TTGT (bold letters). The single base insertion 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 directed repeats
(**TGTGGAATG/G/TGTG*TGAAT***GCCCTGAGT).

Elements such direct and inverted repeats are commonly found in occurrence of gene deletions and single base insertions (Krawczak and Cooper, 1991; Kunkel, 1990). A human consensus sequence was identified as a sequence involved in deletion events and also functioning as an arrest site for polymerase α (Krawczak and Cooper, 1991). This sequence TG(A/G)(A/G)(G/T)(A/C) is often associated with palindromic sequences and also with G+C rich regions. Therefore, arrest of DNA synthesis increases the probability of a slipped mispairing event between direct repeats or the formation of secondary structure intermediates that induce dissociation of the polymerase from the template strand and cause deletions or insertions after reassociation. In our three patients the sequence is close to the mutation sites (see above the nucleotides in italic). In patient A.G. this sequence is present from position 395 to 399, and the mutation occurs 5 bp downstream. In patient N.B. this sequence is detectable from position 36 to 41 (see above) while in the patient A.Z. is present from the nucleotide 1316 to 1321. The same sequence has been found in close proximity to small mutations (<20 bp) in the CYBB gene in almost all X-CGD patients (Roos et al., 1996). Moreover, in this gene single base deletions occur in 78% of the cases in stretches of identical bases while, in the same kind of sequence, 40% of the single base deletion occur. Actually this is the case also for the mutations that we detected in these patients. Accordingly, also in another patient described (Roos et al., 1996) a T deletion occurs at nucleotide 59 within the same TTGT repeat bearing the G deletion in patient N.B.

From all these considerations it is likely that the mutations present in the analyzed patients are generated by the peculiar base sequences present around the mutation sites.

2. Gene therapy for X-linked CGD

As extensively outlined in the Introduction section, CGD is in many terms an ideal candidate disorder for which a gene therapy approach is appropriate. The disease is monogenetic, its phenotype is expressed only in the myelomonocytic hematopoietic lineage, and

the current therapies, including allogenic bone marrow transplantation, are inadequate or unsatisfactory. A further support for the application of gene therapy in this disease comes from the studies in female carriers in which as few as 10% of normal cells give a normal phenotype (Roos et al., 1986). Moreover, patients with a even lower oxidase activity have in some cases a milder clinical course (Roos et al., 1992), suggesting that alternative antimicrobial systems may be important in the outcome of the disease. These observations suggest that correction of only a small percentage of phagocytes can be beneficial for a clinical improvement.

2.1 Construction of and titration of retroviral vectors

In this thesis, I described the construction of four retroviral vectors for gene transfer of gp91-*phox*. The common retroviral backbone of these constructs is the pBabeHygro vector based on MoMuLV (Moloney Murine Leukemia virus) (Morgenstern and Land, 1990). The LTR of this virus are very active in many tissue types but can be inactivated or less functional in other systems like hematopoietic progenitors (Challita and Kohn, 1994). One of the solutions used to overcome this problem is the use of an internal promoter in the retroviral backbone. We used for this purpose the CMV IE promoter, the HV TK promoter and an inducible promoter composed by the TK promoter and an interferon-γ responsive element (Wong et al., 1994). The interferon responsive element was used since the interferon-γ therapy is effective in some case of CGD, probably enhancing the nonoxidative antimicrobial systems of the phagocyte and the locomotion of the cells (see above).

The delivery of exogenous genes using retroviral vectors is an extensively used method for gene transfer into a variety of eukaryotic cell types (Miller, 1992). The production of recombinant amphotropic viruses is usually accomplished by the utilization of packaging cell lines, that allow proper assembly of infectious viral particles bearing the recombinant retroviral genome(Miller et al., 1990). Since the final efficiency of gene transfer depends on the viral titer obtained from the packaging cell cultures, extensive efforts have been made for the improvement of techniques that allow the production of high titer vector stocks (Bunnell et al., 1995; Lynch and Miller, 1991).

The titer of an infectious biological preparation reflects the ability of the produced virus to infect target cells. This property depends on the biological characteristics both of the virus itself and of the target cells. During the process of retroviral infection, there are several key steps that determine the final outcome of infection. In particular, the efficiency depends on the multiplicity at which infection occurs, on the capability of the virus of entering the target cells and being converted in its proviral form, and on the propensity of the proviral molecules of being integrated into, and expressed from, the target cell genome. Monitoring at all these subsequent steps can be exploited to evaluate the viral titer of a packaging cell supernatant.

The most extensively used titration procedure involves the infection of a target cell line (e.g. NIH3T3 fibroblasts) and the subsequent selection for a selectable gene or the detection of a histochemical marker (Miller and Rosman, 1989). Should the retroviral construct not permit any of these two approaches, an alternative possibility is the detection of proviral DNA in the target cell line by Southern blotting (Moore and Belmont, 1993). All these procedures are time consuming, and labor intensive.

Alternatively, provirus formation in the target cell line can be scored by polymerase chain reaction (PCR) (Moore and Belmont, 1993). This method is very sensitive and can be applied after few hours post-infection of the target cell line. However, the results of conventional PCR itself suffer of very poor quantitative reproducibility, being its outcome influenced by several variables that are hardly controllable by the experimenter (Diviacco et al., 1992; Siebert and Larrick, 1992).

For all the above mentioned reasons, we developed a competitive PCR procedure for the screening of packaging cell clones. The developed procedure is very useful for the easy identification of high producers among a high number of cell clones in a very short time. Other methods of titration by PCR suffer of the poor quantitative reproducibility of conventional PCR, require the analysis of serial dilutions of each sample, demand the maintenance of the exponential phase of PCR amplification, and usually require hybridization of the amplification product with an internal probe to increase sensitivity.(Moore and Belmont, 1993)

Besides its rapidity, retrovirus titer determination by quantitative PCR also offers additional advantages over the NIH3T3 colony assay. First, it is highly amenable for the determination of the titer of all recombinant viruses that do not carry a selectable marker. This is a situation that is likely to become very frequent, since the presence of selectable genes is not only useless for the clinical application of gene therapy, but can also be detrimental for optimal virus production (Correll et al., 1994). Furthermore, the determination of proviral titer by the standard colony assay is strictly dependent on the chosen reference cell line and on its culture conditions,(Elefanty and Cory, 1993; Miller and Rosman, 1989) and does not necessarily reflect the actual efficiency of infection of the final cell tissue. In our experience, for example, the retrovirus vectors that are used in this thesis do not score high titers in the murine NIH3T3 assay (~$5x10^4$ for pBabe/Hygro/gp91-*phox*) and nevertheless infects at extremely high efficiency the human hematopoietic progenitor $CD34^+$ cells (more than 80% of colonies scoring positive by DNA-PCR (see results section). The application of competitive PCR for viral titration allows to overcome these discrepancies, since it can be used for monitoring the efficiency of proviral formation in the actual final target tissue of the gene therapy application.

Furthermore, the application of this technique to different cell lines allows its utilization for the determination of the absolute number of transduced cells upon infection with the retroviral vectors. In this case, cells are counted and a known amount of cells are lysed, and the lysate is used in a competitive PCR reaction. The number of proviral molecules detected in the samples allows to initially determine the percentage of transduced cells.

The titers obtained for the four different retroviral vectors are different, being definitely higher for the vector in which expression of the transgene is directly driven by the LTR promoter. This observation clearly reflects the interference that the internal promoters exert on the transcription of the virus driven by the LTR, as also observed by other investigators (Bowtell et al., 1988; Byun et al., 1996; Guild et al., 1988).

2.2 Correction of the genetic defect by retrovirus-mediated gene transfer

The results of the compared activity of the four retroviral constructs in different cell lines such as HeLa and PLB-985 X-CGD and in primary hematopoietic progenitors showed different results. In the HeLa cell line, the LTR are stronger than the other promoters in transcribing the gp91-*phox* gene, while in the myeloid environment the different promoters have roughly the same activity. These data clearly show that, although the internal promoters used are very strong promoters, the influence between the internal promoter and the retroviral LTR is probably influencing the activity of the internal one. This promoter interference has been already demonstrated for other retroviral constructs and reflects complex interactions of multiple promoters. These interactions in some cases can enhance the activity of one promoter, in some other cases instead can shut down completely transcription (Hatzoglou et al., 1991; Vile et al., 1994). It is not clear anyway whether this effect is due to a general promoter interference, probably related to competitive assembly of pol II initiation complexes on the two closely spaced sequences, to a termination effect of the upstream transcripts on the downstream promoter, or to the presence of specific inhibitory sequences in the construct. With respect to the last possibility, it should be noticed that when the gp91-*phox* coding region was cloned in another Moloney vector under the control of the PGK promoter, still transcription was driven by the viral LTR (Kume and Dinauer, 1994).

The effect of promoter interference is even more evident in the vector with the interferon responsive element. In a plasmid the usage of the IRE element upstream the TK promoter resulted in an increased activity upon stimulation with interferon-γ. On the contrary, when cloned within the retroviral backbone, the promoter/enhancer element TK-IRE is not stimulable any more either in epithelial and myeloid cells. Considering that the Moloney LTR promoter is suppressed by interferon-γ (Seliger et al., 1988, and S. Ghazizadeh, "Gene Therapy Meeting", Cold Spring Harbor, 1996, Abstract book p.105) we can deduce that the behaviour of this element in the vector is strongly affected by LTR influence.

It has been already shown that the hormonal stimulation of a promoter can influence in different ways other promoters present in the construct (Hatzoglou et al., 1991) and, in general , that the influence between promoters is not strictly dependent on either their relative strength nor their distance (Emerman and Temin, 1986). Thus, promoter interference is difficult to predict when constructing a retroviral vector.

The ability of the retroviral vector expressing the therapeutic gene from the viral LTR was further tested in EBV-transformed B-cell lines derived from the patients. 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 (Volkman et al., 1984). Success in gene transfer experiments in EBV B-cells has been already achieved for the deficiencies of both the autosomal p47-*phox* (Chanock et al., 1992; Cobbs et al., 1992; Thrasher et al., 1992; Volpp and Lin, 1993) and p22-*phox* (Maly et al., 1993) and for the deficiency of X-linked gp91-*phox* (Porter et al., 1993), using retroviral, episomal or adenoassociated vectors. In B-cell lines a large variability in the efficiency of oxidase function was detected among the different transduced B-cell clones, probably reflecting the different rate of expression resulting from each proviral integration event. The same phenomena has been described for the correction of the oxidase function in the X-CGD PLB-985 (Ding et al., 1996; Kume and Dinauer, 1994). In this case the clonal variation went from 47% to 119% and was not related to the number of proviral integrations. It should be considered, however, that also the EBV-transformed B cell lines from normal individuals show a great variability in the efficiency of O_2^- production, which are likely to be due to variations in the rate of transcription of the gp91-*phox* gene as a response to minor, uncontrollable modifications of cell culture conditions (unpublished observations). Despite this variability, peak values in the range of 20-30% of those of the normal cell clone giving the highest value were obtained in the transduced cell clones. These peak values are equal or even higher than those detected in other B-cell lines from normal individuals. In general, 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. However, the production of O_2^- in some clones (e.g. z10 and z12 in Figure 21 panel A) is higher than the one simply predicted by mRNA expression analysis.

A western blotting analysis on the clone that demonstrated to have the highest oxidase activity showed that the protein was indeed present and that it had the right relative mass. On the other hand, the amount of protein detected is considerably lower than the protein produced by a lymphoblastoid cell line from a healthy individual. The analysis of the mRNA in northern blot revealed that the amount of RNA of some clones is comparable to the amount present in a healthy control, despite that the amount of protein is quite low. The inefficient production of the protein can be caused to different factors. One explanation can be a low efficiency of translation of the viral mRNA. This mechanism has been already suggested also for the results obtained by Ding et al (Ding et al., 1996) in the X-CGD PLB-985 cell line. Whether this effect is linked either to a particular secondary structure of the viral messenger RNA or to the lack of the noncoding region in the gp91-*phox* RNA that prevents an efficient translation of the protein is still not known. Another possible explanation relies on the possible presence of a post-transcriptional block for the accumulation of gp91-*phox* protein in lymphoblastoid cells, probably caused by the limited amount of heme (Chetty et al., 1995).

Despite the low amount of protein translated, the correction of the oxidase activity is in the range of normal B-cell lines. This results are consistent with the results of other authors (Ding et al., 1996; Kume and Dinauer, 1994; Zhen et al., 1993), indicating that even low amount of gp91-*phox* can reconstitute the superoxide activity of the cells. Therefore gp91-*phox* seems not to be rate-limiting for the generation of superoxide. From the standpoint of gene therapy these observations are rather relevant, since a modest amount of recombinant gp91-*phox* protein may lead to a functional reconstitution of the X-CGD phagocytes.

Given the satisfactory results obtained by transduction of B-cell lines with pBabeHygro/gp91-*phox*, we have performed a systematic analysis of the efficiency of this vector for gene transfer of human hematopoietic progenitor cells. For this purpose, the same viral preparation was used in a number of *in vitro* transduction experiments using peripheral blood and bone marrow target cells. These experiments were designed to monitor on the same samples the efficiency of transduction by DNA-PCR, the efficiency of expression by RNA-PCR, and the efficiency of functional correction by the NBT test on differentiated colonies.

The transduction conditions used were based on the infection of hematopoietic progenitor cells with viral supernatant, and allowed us to obtain a reproducibly high level of infection of both unfractionated and $CD34^{+}$-enriched mononuclear cells. The good efficiency of transduction of hematopoietic precursors obtained with this viral preparation, despite its not low titer, is not surprising. As already reported above, it should be considered that the determination of proviral titer by the standard colony assay is strictly dependent on the chosen reference cell line and on its culture conditions, and does not necessarily reflect the actual efficiency of infection of the final cell tissue (Elefanty and Cory, 1993; Forestell et al., 1995; Miller and Rosman, 1989). Additionally, the determination of the efficiency of transduction by measuring the number of DNA-PCR-positive colonies gives a reliable estimate of the actual efficiency of infection of hematopoietic precursors, while selection for drug resistant colonies (as performed in other gene transfer studies) also requires good expression of the resistance gene.

Despite the good efficiency of transduction of the gp91-*phox* gene, functional reconstitution was only partially achieved in about one fifth of the myeloid colonies derived from transduced CGD clonogenic precursors. The variable results obtained by the functional analysis of the colonies are likely to be due to the different rate of expression of the transduced gene, and to the quantitative unreliability of the colorimetric NBT assay. As a matter of fact, the proviral gp91-*phox* gene is probably expressed at low levels in a higher number of colonies, as demonstrated by the increase of the percentage of positive colonies after nested PCR analysis.

2.3 Concluding remarks

The results presented in this work, as well as similar conclusions obtained by different laboratories (Kume and Dinauer, 1994; Li et al., 1994; Porter et al., 1993), indicate that functional correction of X-CGD is feasible by virus-mediated gene transfer. Despite these

promising results, that can be envisioned as a further step toward the genetic correction of the disease, the observed levels of oxidase function in myeloid colonies are probably still below the threshold that could confer an in vivo therapeutic benefit. Accordingly, the evaluation of oxidase function by chemiluminescence assay of transduced $CD34^+$ cells differentiating in liquid culture (performed with the same sample as in Exp. 5 in Table X) gave negative results. Several issues have still to be considered, especially addressing the improvement of the expression of the gp91-*phox* gene in the final target cells. It is likely that the only limited successes obtained so far in terms of efficiency of protein expression could be due to the lack of understanding of the mechanism that control the physiological expression of the gene, in particular in primary cells.

It is noteworthy saying that the conclusions from all the experiments of gene transfer in CGD cells suggest that expression of the transmembrane subunits in a functional form is less efficient than that of the cytosolic components of the NADPH oxidase also using the same expression systems. This might be due to the particular requirement for the assembly of the cytochrome b_{558} that needs the coordinate expression of both subunits and the incorporation of heme and flavin moieties. This necessity can account for the difficulties described so far in correcting cytochrome b_{558} deficiencies.

In this respect, it is encouraging that in our experiments with B-cell clones we have observed a remarkable discrepancy between the relatively modest levels of gp91-*phox* protein and the good degree of functional correction observed in the transduced B-cell lines (as also recently reported by others (Kume and Dinauer, 1994)). This observation suggests that even limited amounts of protein can be sufficient for the assembly of a correct cytochrome b_{558} complex.

It remains still a debatable issue what is the minimal extent of oxidative level that can correct the CGD phenotype. It has been reported that carrier individuals with only 10% of functional phagocytes are perfectly healthy (Roos and de Boer, 1986). However, patients with only a partial reduction of the oxidative function in all the phagocytic cells suffer of the disease (Roos, 1994; Woodman et al., 1995). Accordingly, it should be preferable to obtain high levels of oxidase function even in a minority of cells than partial correction of a majority of cells. This observation again stresses the importance of ameliorating the gene transfer strategy in order to overcome the obstacle of poor expression, for example by different vector design, use of stronger promoters or myeloid-specific enhancers. Only after successful fulfillment of this goal, further problems will need to be addressed, such as the possible in vivo deleterious effects of ectopic expression of the gp91-*phox* protein in other cell lineages (that can be, again, overcome by the use of myeloid specific promoters), or the fate of the transduced cells after transplantation.

Finally, it should be also stressed that, while several ameliorations are envisionable in the near future for the improvement of the efficiency of gene transfer and expression by retroviral vectors, still a major limitations of these vectors is their incapacity of infecting resting cell populations. Thus, it is highly debatable whether successful gene transfer into the primitive

hematopoietic stem cells will be feasible with the current technology for progenitor cell identification and in vitro manipulation. These difficulties are obviously hampering the successful application of gene therapy technologies for CGD, as well as for other diseases in which the curative approach will demand gene transfer in the pluripotent HSC.

The results obtained by the first phase I trial of gene therapy for the autosomal form of CGD due to p47-*phox* defects, conducted by H.L. Malech and collaborators, emphasize and exemplify these problems (International Symposium: "Gene Therapy". Trieste, Italy. 1996. Abstract book p. 26). Neutrophils exhibiting functional correction of oxidase activity started being detectable in peripheral blood of patients who received transfusion of retrovirally-engineered $CD34^+$ cells at 3-4 weeks after infusion, at a frequency of 0.005-0.05%, and lasted for several months. This delayed appearance of corrected cells followed by a several months persistence is evidence of a low level of engraftment of corrected early progenitors.

Given these considerations, it is clear that gene therapy of CGD will definitely benefit from the desirable progresses that will be made in the future times in the understanding of the real nature and properties of pluripotent HSC, and on the development of satisfactory protocols for gene transfer in these cells. Several topics of investigation appear suitable for further and deeper investigation:

i) Control of HSC renewal in normal resting cells is poorly understood at present but it is assumed to be controlled by the range and concentrations of hematopoietic growth factors in extracellular matrix surrounding bone marrow stromal cells (Metcalf and Nicola, 1991; Nolta and Kohn, 1990). Although the conditions for transduction of PHSC are not yet completely standardized, many improvements have been done to understand the effect of lymphokines used during the infection protocols and the conditions that can preserve the renewal capacity of the stem cells. Recent studies using stromal cell support have enhanced the efficiency with which this can be attained by retroviruses using long-term bone marrow culture in vitro (Nolta et al., 1996; Nolta et al., 1995; Xu et al., 1994).

ii) A major problem that compromises the field in its approaches to the transduction of pluripotent HSCs is that there are neither phenotypic markers available for these cells, nor reliable in vitro assays. Assays for colony-forming, cytokine-responsive cells or even for long-term culture initiating cells (LTC-IC) are surrogate in vitro assays that do not correlate perfectly with the capacity of animal marrow reconstitution. Furthermore, in human and non human primates reconstitution studies are either ethically prohibited or too costly to be widely applicable. A reasonable experimental model which starts to be commonly considered as a reliable assay for PHSC is the human-SCID or NOD-SCID mice, in which human PHSC are scored for their capacity to reconstitute hematopoiesis in immunodeficient mice (Larochelle et al., 1996; Nolta et al., 1996; Salomon et al., 1990; Shultz et al., 1995; Vormoor et al., 1994)). However, even in this case, perfect correlation to human or non-human primate marrow reconstitution still remains to be

completely demonstrated. Further developments in this area will prove to be useful in judging the transduction efficiency of pluripotent HSCs.

iii) A number of experimental and clinical considerations point out that, in terms of engraftment and to ensure repopulation of the recipient with the transduced cells, bone marrow ablation of the recipient can dramatically increase the repopulating ability of PHSC. However, ablation of bone marrow is not a desirable option in gene therapy protocols for inherited disorders of blood cells, particularly when patients are already immunocompromised. Therefore, and in the absence of methods for in vitro expansion of PHSC, the development of vectors containing additional genes which confer a selective advantage in vivo to the transduced cells is highly desirable. Among these genes, those conferring resistance to chemotherapeutic agents appear to be the most appealing, such as the mdr-1 (Sorrentino et al., 1995) or the DHFR (Li et al., 1994) genes.

iv) Given the refractoriness of HSCs to be transduced by retroviral vectors, novel strategies for gene transfer in non cycling cells are desirable. Particularly attractive gene delivery systems are those based on the adeno-associated virus (AAV) and on lentiviruses. These viruses have many attributes that render them attractive for gene therapy of HSCs. However, their wider application for gene therapy will require further and deeper investigations about their molecular biology features.

Given all the above mentioned considerations, it can be concluded that the road to the successful development of gene therapy of hematopoietic stem cells still needs several experimental goals to be accomplished. With this respect, CGD represents an ideal model disorder to address a number of the still unresolved issues.

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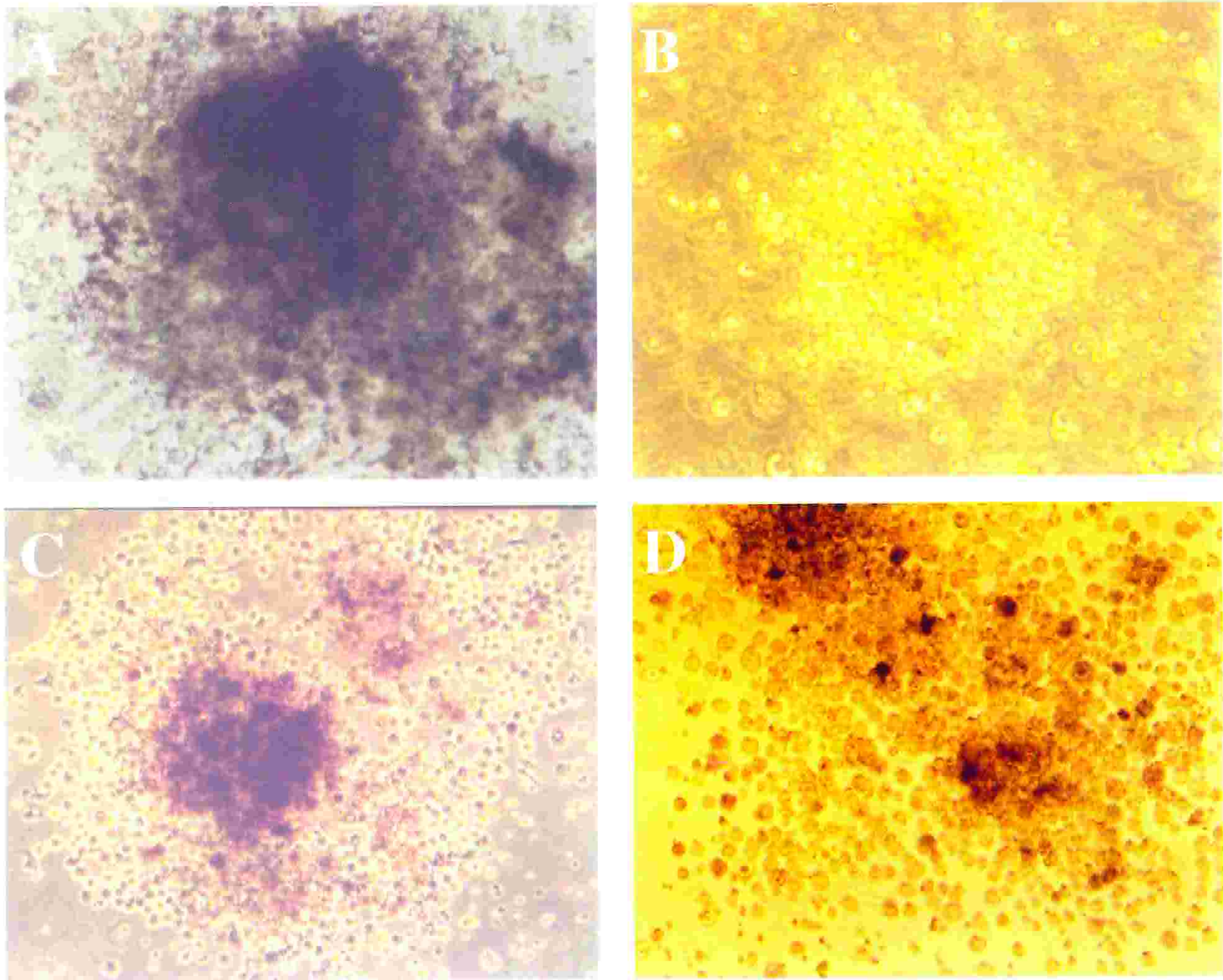

Figure 24. Functional reconstitution of NADPH oxidase activity in hematopoietic precursors.

(A) Nitroblue tetrazolium (NBT) assay on an in vitro-differentiated myeloid colony from peripheral blood hematopoietic precursors of a normal individual. The dark blue staining indicates reduction of the dye by superoxide

(B) NBT assay on a myeloid colony from a CGD patient, with no evidence of oxidase activity.

(C, D) NBT assay on two myeloid colonies from a CGD patient after retroviral gene transfer, using the pBabeHygro/gp91-phox virus; the colonies show partial functional correction of the defect.