Molecular Studies Towards Gene Therapy for Chronic Granulomatous Disease

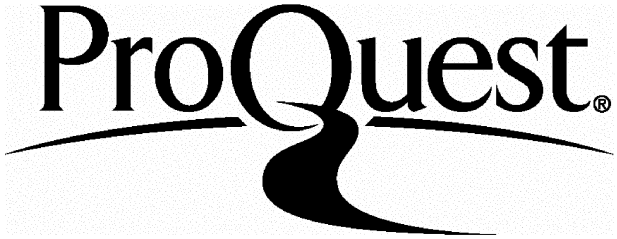
by

Adrian James Thrasher

Department of Medicine
University College London School of Medicine

1995

A thesis presented for the degree of Doctor of Philosophy



ABSTRACT

Chronic granulomatous disease (CGD) is human disorder arising from heterogeneous molecular lesions in the genes encoding a phagocyte specific enzyme system, the NADPH-oxidase. The importance of this system to host immunity is exemplified by the clinical phenotype arising from its defective function, which is characterised by recurrent bacterial and fungal infection. Bone marrow transplantation can be curative, but the paucity of suitable donor material, and the increased risks and complications associated with use of matched unrelated donor grafts, restricts application of this procedure to a minority of cases.

CGD is an excellent candidate disorder for the development of somatic gene therapy. The studies presented in this thesis investigate potential mechanisms for determination of the myeloid and differentiation-specific expression of one of the NADPH-oxidase components, $p47^{phox}$, and demonstrate the applicability of a gene transfer approach using recombinant retrovirus-based systems to permanently reconstitute NADPH-oxidase activity in immortalised B cells. However, the current generation of retroviral vectors are limited by inefficient transduction of relatively quiescent cells, which include the pluripotent haematopoietic stem cell population. This greatly restricts their potential to effect lifelong correction of the clinical phenotype. One system which may be less dependent on cell cycle for successful transduction is based on the dependovirus, adeno-associated virus (AAV). These vectors are shown to stably transduce and restore NADPH-oxidase activity to patient-derived B cells, but are also shown to be difficult to propagate. A method for rescue of recombinant AAV particles from replication-defective adenoviruses is described, which facilitates production, and significantly reduces the risk of contamination with wild-type adenovirus. Adenoviral vectors are also shown to restore function to primary monocytes derived from patients, and may prove to be useful for delivery of the recombinogenic AAV vector genome to target cells.

ACKNOWLEDGMENTS

I would like to thank all those in the laboratories at the Department of Medicine, University College London, and at the Molecular Immunology Unit of the Institute of Child Health for help and encouragement throughout the course of this work. I am particularly grateful to Colin Casimir for his informed advice, and for numerous helpful discussions without which this work would not have been possible. For similar reasons, I must thank Christine Kinnon, Mary Collins, Colin Porter, Gordon Stewart, and Jane Lebkowski. I am grateful to the Molecular Pathology Unit at the Middlesex Hospital for synthesis of oligonucleotides, and to Mahesh de Alwis for technical assistance in the purification of viral preparations.

I am primarily indebted to Tony Segal for introducing me to the world of molecular medicine, and to both Tony, and Roland Levinsky for unfailing encouragement, support, and confidence in my ability.

This thesis is dedicated to my wife Kanchan, for undying support, and to our daughter, Maya, for helpful distraction during the writing process.

TABLE OF CONTENTS

CHAPTER 2
MATERIALS AND METHODS

CHAPTER 3
THE PROMOTER REGION OF THE p47^{phox} GENE

CHAPTER 4
RETROVIRUS-MEDIATED GENE TRANSFER

CHAPTER 5
ADENO-ASSOCIATED VIRUS GENE TRANSFER

CHAPTER 6
FACILITATING RECOVERY OF rAAV PARTICLES

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAV	adeno-associated virus
AAVS1	AAV integration locus
Ad	adenovirus
ATP	adenosine triphosphate
bp	base pairs
BES	N'N-bis[2 hydroxyethyl]-2-aminoethanesulphonic acid
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAT	chloramphenicol acetyl transferase
CD	cluster designation
CDP	CCAAT-displacement protein
cfu	colony forming units
CGD	chronic granulomatous disease
CMV	cytomegalovirus
COUP	chicken ovalbumin upstream promoter
CFU-C	colony forming units
CSF	colony stimulating factor
CYBA/B	genetic loci for $p22^{phox}$ /$gp91^{phox}$ genes
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
E1-4	adenovirus early region 1-4
EBV	Epstein Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol-bis[β-aminoethyl ether]N,N,N',N'- tetra acetic acid
FAD	flavin adenine dinucleotide
FCS	foetal calf serum
fMLP	formyl-methionyl-leucyl-phenylalanine
G-CSF	granulocyte colony stimulating factor
G6PD	glucose-6-phosphate dehydrogenase
GDI	GDP-dissociation inhibitor
GDP/GTP	guanosine di/triphosphate
GDS	GDP-dissociation stimulator
Glvr-1	gibbon ape leukaemia virus receptor
GM-CSF	granulocyte-macrophage colony stimulating factor
GvHD	graft versus host disease
HEPES	N'[2-hydroxyethyl] piperazine-N'-2-ethanesulphonic acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HLH	helix-loop-helix
IFN	interferon
Il	interleukin
ISRE	interferon-stimulated response element
LacZ	gene encoding β-galactosidase

LCR	locus control region
LPS	lipopolysaccharide
LTC-IC	long term culture initiating cell
LTR	long terminal repeat
MAR	matrix attachment site
M-CSF	macrophage colony stimulating factor
MEM	modified Eagle's medium
MHC	major histocompatability complex
MLV	murine leukaemia viruses
moi	multiplicity of infection
MoMLV	Moloney MLV
MPSV	myeloproliferative sarcoma virus
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
NCF-1/2	genetic locus for $p47^{phox}$ / $p67^{phox}$
NK	natural killer cell
NP-40	nonidet P-40
nt	nucleotides
ORF	open reading frame
PAF	platelet activating factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque-forming units
phox	phagocyte oxidase
PHSC	pluripotent haematopoietic stem cell
PKC	protein kinase C
PMA	phorbol myristate acetate
PMSF	phenyl methyl sulphonyl fluoride
PRS	purine rich sequence
RA	all trans retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RRS	Rep protein recognition sequence
RSB	reticulocyte standard buffer
RXR	retinoid X receptor
SINE	short interspersed nuclear element
SCF	stem cell factor
SCID	severe combined immunodeficiency
SOD	superoxide dismutase
SDS	sodium dodecyl sulphate
SH3	*Src*-homology 3
Tag	SV40 large T antigen
TLCK	Nα-p-tosyl-L-lysine cloromethyl ketone
TNF	tumour necrosis factor
TR	(inverted) terminal repeat
TREpal	thyroid hormone response element

trs	terminal resolution site
Tris	Tris[hydroxymethyl]amino-methane
VDR	vitamin D receptor
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER ONE:

CHRONIC GRANULOMATOUS DISEASE

1.1 INTRODUCTION

A clinical syndrome characterised by recurrent life threatening sepsis, hypergammaglobulinaemia, and widespread chronic granulomatous infiltration was first recognised in the paediatric literature between 1954 and 1960 (Janeway and Gitlin, 1954; Landing and Shirkey, 1957; Berendes et al. 1959; Quie, 1993). The pathological mechanisms responsible for this condition became evident when it was demonstrated that neutrophils collected from a male patient were unable to kill *Staphylococcus aureus in vitro*, and that there was a primary abnormality of neutrophil function (Quie et al. 1967). In the same year, it was shown that neutrophils from patients with this familial granulomatosis, now called chronic granulomatous disease (CGD), failed to exhibit a characteristic increase of oxidative metabolism during phagocytosis called the 'respiratory burst' (Holmes et al. 1967).

1.2 THE NADPH OXIDASE

1.2.1 The substrate NADPH

The enhanced 'respiration' of leukocytes was first described as a small but significant increase in the oxygen consumption of canine neutrophils during phagocytosis of bacteria (Baldridge and Gerard, 1933). This metabolic response was initially attributed to increased generation of energy during phagocytosis, and was known as 'the extra respiration of phagocytosis', but was later shown to be resistant to conventional inhibitors of mitochondrial respiration (Sbarra and Karnovsky, 1959). It was also shown that the necessary energy for phagocytosis and cytoplasmic degranulation was provided by the glycolytic pathway. The function of the 'respiratory burst' remained obscure until it became apparent that the ability of phagocytic cells to kill certain bacteria *in vitro* was markedly diminished under anaerobic conditions, and that cells obtained from patients with CGD, which were unable to mount this metabolic response,

exhibited the same microbicidal deficiency in the presence of oxygen (Quie et al. 1967). Together, these observations indicated a crucial function for this system in host defence, although the mechanisms by which the respiratory burst mediate microbial killing remain the subject of some debate.

The identity of the substrate for the reaction was the subject of considerable speculation (Baehner and Karnovsky, 1968; Segal and Peters, 1976; Badwey and Karnovsky, 1979), but the sharp increase in oxidation of glucose via the hexose monophosphate shunt (the purpose of which is to maintain cellular NADPH levels, and the activity of which is controlled by the rate of oxidation of NADPH) co-incident with neutrophil activation, strongly suggested that NADPH was the most likely candidate molecule (Anonymous, 1964). In support of this, patients with severe glucose-6-phosphate dehydrogenase (G6PD) deficiency, in whom cellular replenishment of NADPH is minimal, show markedly diminished consumption of oxygen in response to stimulation (Baehner et al. 1972; Cooper et al. 1972; Gray et al. 1973).

1.2.2 Discovery of a unique cytochrome component of the NADPH-oxidase

A cytochemical staining reaction produced by the conversion of a mixture of para-phenylene diamine and α-napthol to the blue compound indophenol-blue was first described in 1961 (Hattori, 1961). This reaction was originally attributed to cytochrome activity present in mitochondria, but could not account for the particularly strong reaction found in neutrophil leukocytes, which was shown to be specifically associated with neutrophil granules. On the basis of this, several workers identified a b-type cytochrome, cytochrome b_{558} (based on the absorption maximum of the α-band in the oxidised-minus-reduced difference spectrum (Hattori, 1961; Cross et al. 1982a)), which bound carbon monoxide (CO), and which could be reduced by NADH and NADPH under anaerobic conditions (Shinagawa et al. 1966). A close relationship with previously identified phagocyte NADPH-oxidase activity was likely (Rossi and Zatti, 1964; Ohta et al. 1966), although the molecular basis remained obscure until the same cytochrome b_{558} was shown to be missing from neutrophils of some patients with CGD, and was finally identified as the terminal electron transporting component of the NADPH-oxidase (Segal and Jones, 1978). The midpoint potential for this cytochrome

(–245mV) is the lowest recorded for any mammalian cytochrome b and is low enough to induce direct reduction of oxygen to superoxide (Wood, 1987). It is therefore sometimes designated cytochrome b_{-245}.

1.2.3 Purification of cytochrome b_{558}

Purification of the cytochrome b_{558} and identification of its constituent polypeptides proved difficult predominantly as a result of an abundance of proteolytic enzymes within neutrophil granules, and anomalous behaviour of the heavily glycosylated β-subunit on SDS/polyacrylamide gel electrophoresis (SDS/PAGE) (Harper et al. 1984; Pember et al. 1984; Serra et al. 1984; Lutter et al. 1985). Two reliable methods now exist for the purification of this cytochrome (Harper et al. 1984; Parkos et al. 1987), both of which isolate two proteins with apparent molecular weights on SDS/PAGE of 23 kDa (α-subunit) and 76-92 kDa (β-subunit) respectively. The larger β-subunit migrates on SDS/PAGE as a broad band, an electrophoretic property characteristic of glycoproteins, and comprises about 21% carbohydrate, predominantly of the N-linked high-lactosamine complex type (Harper et al. 1985). Although an intimate relationship between these two molecules has been established by their co-purification, the precise stoichiometry of the subunits in the active complex is unknown. Both proteins are missing in cells derived from most CGD patients with a molecular lesion of either subunit, indicating that mutual interaction is necessary for stability of the mature complex (Parkos et al. 1987; Segal, 1987; Parkos et al. 1989). A 65kDa biosynthetic $gp91^{phox}$ intermediate precursor with high-mannose type oligosaccharide side chains has been detected in membrane fractions (which includes endosomal compartments) derived from patients with $p22^{phox}$-deficient CGD, which is processed to a mature terminally glycosylated form only if the deficiency of the smaller subunit protein is restored (Porter et al. 1994). This may suggest a requirement for mutual association prior to final modification in the Golgi.

1.2.4 Identification of substrate and co-factor binding sites

The location of the binding site for the substrate NADPH was unknown until the photoaffinity ligand 2-azido NAD(^{32}P)H was shown to identify a membrane protein of approximately 93kDa, in the region of the large subunit of the cytochrome b_{558} (Segal

et al. 1992). Significantly, the same protein could not be identified in 3 X-linked CGD patients known to lack cytochrome b_{558}, and in a single patient with a proline to histidine mutation at a putative NADPH binding site, in whom immunoreactive membrane bound cytochrome was detectable.

The nucleotide binding protein of electron transport chains is almost invariably a flavoprotein, and it was therefore probable that an FAD containing flavoprotein was an essential component of the NADPH-oxidase. Involvement of a flavoprotein was postulated many years ago (Cagan and Karnovsky, 1964), but identification of the specific NADPH-binding flavoprotein that transports electrons from the substrate to the cytochrome b_{558} eluded identification until recently, largely because FAD becomes detached from the apoprotein upon detergent solubilisation of the membranes. Several lines of evidence now indicate a primary location for the flavoprotein in the membrane (Kakinuma et al. 1986; Chiba et al. 1990), and are supported by the observation that membranes purified from patients with X-linked cytochrome-negative CGD have grossly diminished levels of FAD compared with membranes fractionated both from normal cells, and from patients with autosomal recessive CGD, in whom levels of functional immunoreactive cytochrome are normal (Cross et al. 1982b; Gabig and Lefker, 1984; Bohler et al. 1986; Ohno et al. 1986; Segal et al. 1992). Direct association of FAD with the cytochrome is indicated by cell-free experiments in which purified cytochrome, re-lipidated in the presence of FAD, retained activity and by implication FAD, after gel filtration (Rotrosen et al. 1992). The ratio of FAD to haem has been estimated to be 1:2, and suggests a mechanism by which the two electrons donated by NADPH and carried by FAD could be separately donated to oxygen to form superoxide, each being transported by one of the two haems (Segal et al. 1992).

The experimental evidence outlined above is now supported by the identification of strong sequence homology to distinct regions of the ferredoxin reductase family of proteins, and an emerging consensus has identified the β-subunit of the cytochrome itself as the location of the flavin and NADPH binding site (Porter, 1991; Dancis et al. 1992; Segal et al. 1992; Rotrosen et al. 1992a; Sumimoto et al. 1992; Taylor et al. 1993). Cytochrome b_{558} is therefore a flavocytochrome b, the first to be described in

higher eukaryotic cells, and comprises the complete electron transporting apparatus of the NADPH-oxidase.

The location and number of haem molecules within the NADPH-oxidase complex remains unresolved. Previous models have favoured association with the α-subunit, probably liganded to two histidines, but is contradicted by the observation that one of the two His residues on the α-subunit (His72) is the site of a polymorphism that does not affect haem binding (Dinauer et al. 1990). In contrast, His93 is within a region of homology (V-L-H-L) shared with the haem binding subunit of cytochrome c oxidase (Parkos et al. 1988). Two CGD patients have been identified in whom mutations occur at different histidine residues within the β-subunit, but because protein is undetectable, instability unrelated to binding of haem cannot be excluded (Bolscher et al. 1991). Haem may therefore be bound to the α-subunit by a sixth ligand alternative to His, between two α-subunits, or shared between both α and ß-subunits (Quinn et al. 1992).

1.2.5 Association with Rap1A

The small GTP-binding protein Rap1A, is a member of the Ras superfamily of GTP-binding proteins with about 50% homology to human K-Ras, but less than 30% homology with Rac1/2 (see below) and has been implicated in the function of the NADPH-oxidase. Rap1A was found by N-terminal sequencing to co-purify with the flavocytochrome (Quinn et al. 1989), and by implication to exist in the membrane physically associated with both subunits. Most reports suggest that Rap is permanently resident in membrane fractions (Maridonneau-Parini and de Gunzburg, 1993; Quinn et al. 1993), but some have suggested that it is translocated to the membrane from the cytosol upon activation of the cell (Eklund et al. 1991). Affinity for the flavocytochrome is modulated by cAMP-dependent phosphorylation of Rap, under which conditions the association seems to be decreased (Bokoch et al. 1991; Quilliam et al. 1991). This has led to suggestions that Rap may control the attenuation of the respiratory burst in response to agonists which elevate cAMP. Attempts to reconstitute activity in a cell-free system using recombinant Rap have had mixed results (Eklund et al. 1991; Knaus et al. 1992), and the evidence that Rap1A participates in the NADPH-oxidase system is for the moment based on co-purification with the flavocytochrome.

1.2.6 The cell-free system

The 'cell free' system is an *in vitro* assay for NADPH-oxidase activity induced in broken cell preparations, and has provided an important tool for the elucidation of many aspects of oxidase biology. When the substrate NADPH is added to broken preparations of resting cells no activity is detectable. However, if the cells are activated prior to being disrupted, oxidase activity can be detected in the homogenate. A number of groups found that they could induce activity in a homogenate from inactive cells by the addition of amphiphiles such as arachidonic acid or sodium dodecyl sulphate, and forms the basis of the cell-free reaction system (Bromberg and Pick, 1984; Bromberg and Pick, 1985; Curnutte, 1985; McPhail et al. 1985).

The first indication that cytosolic factors were necessary for activation of the NADPH-oxidase came from cell-free studies on whole cell homogenates (Bromberg and Pick, 1984; Heyneman and Vercauteren, 1984; Curnutte, 1985; McPhail et al. 1985). Somewhat surprisingly, the particulate fraction of the homogenate, which was believed to contain the membrane bound NADPH-oxidase, could not be activated unless the soluble fraction consisting predominantly of cytosol was also present (Curnutte et al. 1987; Babior et al. 1988). Furthermore, some patients with CGD were deficient in this cytosolic activity (Curnutte et al. 1988), and although normal levels of functional cytochrome b_{558} were detected (Segal et al. 1983), passage of electrons from the substrate NADPH to haem did not occur (Segal and Jones, 1980). This suggested an abnormality of the activation process or the absence of proximal electron-transferring components.

1.2.7 Cytosolic components of the NADPH-oxidase

The first clearly defined biochemical abnormality to be noted in autosomal recessive CGD was a failure to phosphorylate a 47kDa cytosolic protein when neutrophils were stimulated by phorbol myristate acetate (PMA), an activator of protein kinase C (Segal et al. 1985). The cloning and sequencing of the gene coding for this protein, and that of another cytosolic component of the oxidase, resulted from a serendipitous experiment in which a polyclonal antiserum recognised two proteins, a 47kDa protein which was

absent in most patients with autosomal recessive CGD (AR-CGD), and a 67kDa protein that was missing in a few other cases with a similar inheritance (Volpp et al. 1988). According to standard nomenclature in which the superscripted suffix ^{phox} denotes 'phagocyte oxidase', these proteins are known as p47^{phox} and p67^{phox}. Complementation between cytosolic fractions derived from CGD patients has shown that AR-CGD results in the majority of cases from the absence of either p47^{phox} or p67^{phox} (Nunoi et al. 1988; Volpp et al. 1988; Bolscher et al. 1990; Kenney et al. 1990). Phosphorylation of both proteins coincides with activation of the NADPH-oxidase, but the functional significance is unclear, and is not necessary for cell-free activation (Segal et al. 1985; Heyworth and Segal, 1986; Caldwell et al. 1988; Kramer et al. 1988; Okamura et al. 1988; Bolscher et al. 1989; Kramer et al. 1989; Dusi et al. 1993).

Fractionation of cytosol by various chromatographic methods has now isolated several factors which act synergistically to activate the oxidase in the cell free system (Fujita et al. 1987; Curnutte et al. 1988; Nunoi et al. 1988; Pick et al. 1989; Bolscher et al. 1989; Bolscher et al. 1990). One of these, p21*rac*1, was co-purified with rho-GDI (GDP-dissociation inhibitor) from a cytosolic fraction prepared from guinea pig macrophages (Abo et al. 1991; Abo and Pick, 1991), and was found to be essential for cell-free activation of the NADPH-oxidase. Antisense oligonucleotides directed against *rac* have subsequently been shown to reduce both the amount of immunoreactive *rac* protein, and to produce a dose dependent inhibition of superoxide production in whole cells (Dorseuil et al. 1992). This small G-protein is a member of the Ras superfamily of GTP-binding proteins, with which it shares approximately 30% homology. Somewhat surprisingly, in view of its proposed specific role in NADPH-oxidase activation, p21*rac*1 has a wide tissue distribution, and it is possible that p21*rac*2, which shares 92% amino acid identity with p21*rac*1, and which is expressed predominantly in cells restricted to myeloid lineages, is the physiologically active molecule (Didsbury et al. 1989; Moll et al. 1991; Reibel et al. 1991; Knaus et al. 1992). The physiological function of *rac* remains obscure, but probably involves cycling between an inactive GDP-bound state, and an active GTP-bound state in which it can mediate the assembly or activation of the NADPH-oxidase (Segal and Abo, 1993). These events may be

modulated by GTPase activity outside that of *rac* itself, and by other factors such as GDP-dissociation inhibitor (GDI), and GDP-dissociation stimulator (GDS).

The latest addition to the cluster of cytosolic oxidase factors is a 40 kDa protein, $p40^{phox}$ which was found to co-purify with $p47^{phox}$ and $p67^{phox}$ following immunoprecipitation or purification of these proteins by column chromatography (Someya et al. 1993; Wientjes et al. 1993). In view of the stable association between $p67^{phox}$ and $p40^{phox}$ during purification, and the observation that levels of $p40^{phox}$ are greatly diminished in $p67^{phox}$-deficient neutrophils, the primary association is probably between $p40^{phox}$ and $p67^{phox}$. It is therefore possible that for some patients with $p67^{phox}$ deficient AR-CGD the primary lesion affects $p40^{phox}$, resulting in a secondary instability of $p67^{phox}$.

1.3 ACTIVATION OF THE NADPH-OXIDASE

1.3.1 Activation stimuli

The oxidase remains inactive until exposed to appropriate stimuli, after which there is a short lag phase preceding an increase in oxygen consumption of up to one hundred fold, depending on the cell type, and the stimulus used (Badwey et al. 1980; Karnovsky and Badwey, 1986). The classical stimulus of the respiratory burst *in vivo* is a particle opsonised with immunoglobulin and/or complement, which attaches to specific receptors on the plasma membrane. This triggers phagocytosis and vacuolar closure before localised activation of electron transport across the wall of the vacuole, and the formation of superoxide within the lumen. A variety of physiological receptor stimuli have been used in experimental systems to study the oxidase (Baggiolini et al. 1993). These include the complement fragment C5a, N-formylated methionylpeptides derived from micro-organisms, bioactive lipids such as platelet activating factor (PAF) and Leukotriene B4 (LTB4), and several neutrophil activating proteins including IL-8 (Rossi, 1986; Morel et al. 1991; Cross and Jones, 1991). Many artificial stimuli have also been applied, most commonly direct agonists of PKC such as the phorbol ester phorbol myristate acetate (PMA). The detail of activation mechanisms involved in these processes remain unclear.

1.3.2 Translocation of cytosolic components to the membrane

The flavocytochrome b_{558} almost certainly comprises the complete electron transporting system, and either itself or an associated molecule, forms the membrane docking site for the cytosolic components. In resting neutrophils, the plasma membrane is devoid of flavocytochrome which resides almost exclusively in specialised light density intracellular vesicles and within the membranes of specific granules (Garcia and Segal, 1984; Sengelv et al. 1992). When the cell is activated the plasma membrane invaginates to form the phagocytic vacuole with which vesicles containing flavocytochrome b_{558} fuse. Specific cytosolic components form an activation complex which translocates to the membrane to associate with and induce electron transport through the flavocytochrome b_{558} (Fig1.1). Translocation of both $p47^{phox}$ and $p67^{phox}$ proteins depends on the presence of $p47^{phox}$ and membrane-bound flavocytochrome b_{558} (Clark et al. 1990; Heyworth et al. 1991).

1.3.3 Induction and termination of electron transport

It has become possible to reconstitute NADPH-oxidase activity *in vitro* with purified relipidated flavocytochrome b_{558}, and recombinant $p67^{phox}$, $p47^{phox}$, and p21*rac1* (in the GTP-bound state), or purified cytoplasmic *rac*2/GDI complex (Abo et al. 1992; Rotrosen et al. 1992a; Rotrosen et al. 1992b). For these experiments, there is an absolute requirement for the addition of exogenous FAD. More recently, reconstitution has been achieved using recombinant flavocytochrome b_{558} purified from a haem-supplemented baculovirus system (Rotrosen et al. 1993), indicating that all co-factors must occupy binding sites within these defined proteins, and most probably as discussed above, on the flavocytochrome b_{558} itself. Assembly of the complete NADPH-oxidase complex may induce conformational changes in flavocytochrome b_{558} which permit binding of the substrate NADPH, and which are conducive for electron transport. Other proteins, for example $p40^{phox}$, are not essential in this artificial situation, and are therefore more likely to be important for the stabilisation of individual component proteins and for their initial assembly into an activation complex at the cell membrane.

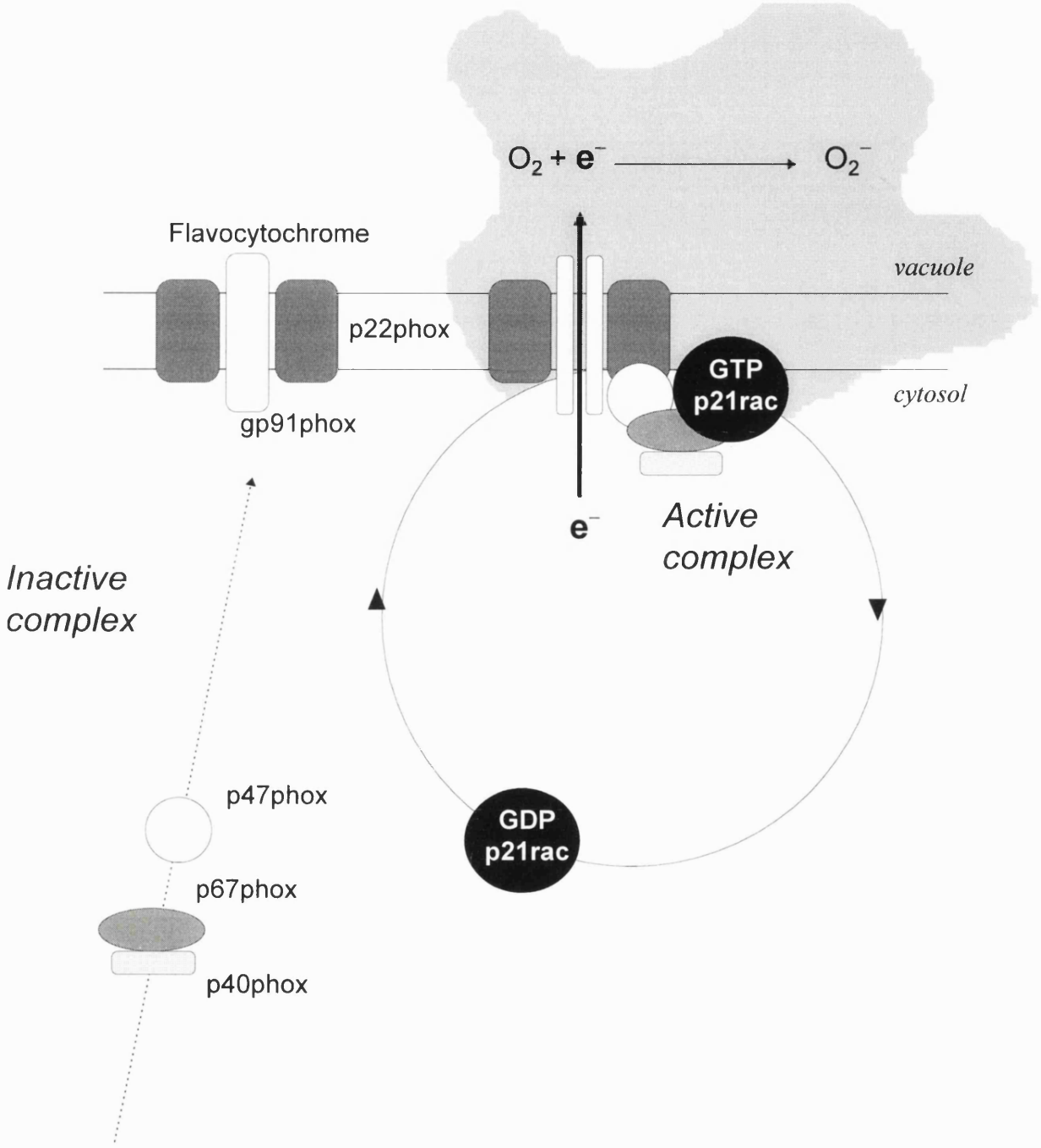


Fig 1.1. Schematic activation of the NADPH-oxidase. When the phagocytic cell is activated, the plasma membrane invaginates to form a phagocytic vacuole, and intracellular vesicles containing the flavocytochrome fuse with the plasma membrane, which in the resting state is relatively devoid of this complex. Induction of electron transport depends on translocation of the cytosolic components, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and p21*rac* and interaction with the membrane-bound flavocytochrome. Interaction between a proline-rich region on the cytoplasmic domain of $p22^{phox}$ with SH3 domains of $p47^{phox}$ appears to be obligatory, but may not be the only physical interaction. The phosphorylation status of the guanine nucleotide bound to p21*rac*, may be essential for induction and termination of activation.

NADPH-oxidase activity occurs transiently after phagocytosis and is dependent upon continued receptor occupancy, and continued association of oxidase components at the membrane. Termination of the response is not simply mediated by the release of $p47^{phox}$ and $p67^{phox}$ into the cytosol as these remain in association with the membrane well after the burst is over (Dusi et al. 1993). One potential regulator of termination is the GTP-binding protein p21*rac*, the activity of which appears to be governed by the phosphorylation state of the guanine nucleotide. GTP-binding protein regulatory proteins such as GDP dissociation stimulator (GDS) and inhibitor (GDI), and GTPase activity within other components of the activation complex may play important roles in the activation and termination of activity. More recently it has been suggested that protein kinase C (PKC) and type 1 and 2A protein phosphatases are intimately involved in a continuous phosphorylation reaction that maintains the oxidase in an assembled and active state (Curnutte et al. 1994).

1.3.4 *Src*-homology 3 domains

Src-homology 3 (SH3) domains are composed of conserved sequences of 50-60 amino acids which are important for protein-protein interaction, often mediated through proline rich sequences in target proteins (Musacchio et al. 1992). Two such SH3 domains are found in both $p47^{phox}$ and $p67^{phox}$ and are necessary for NADPH-oxidase activation in whole cells. Interestingly, $p67^{phox}$ mutant proteins lacking both SH3 regions are fully active in cell-free systems. Function of the single domain within the $p40^{phox}$ sequence has not been investigated.

The possible functions of SH3 domains in this system are clearly numerous, and may involve binding of the cytosolic components to the membrane, to the submembranous cytoskeleton, or to other components of the NADPH-oxidase, including the flavocytochrome b_{558}. Recent experimental evidence supports a mutual interaction between SH3 domains and proline-rich sequences on both $p47^{phox}$ and $p67^{phox}$, interaction between SH3 domains in $p47^{phox}$ and three proline-rich domains in the C-terminus cytoplasmic region of $p22^{phox}$, and intramolecular association of SH3 regions in $p47^{phox}$ and proline-rich sequences within the same molecule (McPhail, 1994). A P156Q mutation in the middle proline-rich region of $p22^{phox}$ resulted in markedly

reduced binding of a synthetic fusion protein containing both p47 ^{phox} SH3 domains, and synthetic peptides containing this middle region abolish binding between wild type sequences (Leto et al. 1994). Furthermore, neutrophils derived from a CGD patient with a natural P156Q mutation expressed normal levels of non-functional flavocytochrome, which nonetheless was permissive for electron transport in a cytosol-independent cell-free activation system (Leusen et al. 1994). In activated whole cells and in a cytosol-dependent cell-free system, translocation of cytosolic factors did not occur, suggesting that the proline-rich region on the membrane-bound p22^{phox} mediates an essential interaction with cytosolic factors.

Independent of their participation in protein-tyrosine kinase-linked receptor pathways, SH3 domains have been found in a diversity of other proteins, including some of those that constitute, or are associated with the cytoskeleton. Recently, it has been suggested that the function of SH3 domains extends beyond passive protein-protein interaction, to include interactive regulation of small GTP-binding proteins (Fry, 1992; Gout et al. 1993), and targeting of signalling molecules to specific subcellular locations (Bar-Sagi et al. 1993). These observations are clearly of importance in view of the obligatory participation of p21*rac* in the activation of the NADPH-oxidase, and it is conceivable that SH3 domains of the cytosolic factors are intimately involved in the regulation of *rac* function.

1.3.5 Interaction with the cytoskeleton

There is some evidence for interaction of components of the NADPH-oxidase with the cytoskeleton, although the nature of this interaction is unclear. Experimentally, p67^{phox} and to a lesser extent p47^{phox} (Nauseef et al. 1991; Woodman et al. 1991), are associated with the detergent-insoluble fraction when neutrophils are permeabilised with Triton-X100. Cytoskeletal involvement is further implicated by experimental observations that *rac,* and related *rho* proteins, when injected into the cytosol of fibroblasts in the active GTP-bound form, control cytoskeletal events such as membrane ruffling and formation of actin stress fibres in response to growth factors (Hall, 1992; Ridley et al. 1992). These small GTP-binding proteins are isoprenylated at their C-termini, by the attachment of geranyl-geranyl (C20) chains in the case of *rac* 1 and 2, to a sequence of

amino acids known as the CAAX box (Cysteine-Aliphatic-Aliphatic-X) (Hall, 1992). This lipid tail may be important for interaction with membranes, and may be required for the translocation of the other cytosolic proteins, with or without a cytoskeletal attachment. The exact role of the cytoskeleton in the operation of this system remains uncertain, and is not a requirement for activation in a cell-free situation.

1.4 FUNCTION OF THE NADPH-OXIDASE

1.4.1 NADPH-oxidase activity in different cell types

The NADPH-oxidase is most abundant in phagocytic cells, namely neutrophils, eosinophils and cells of the monocyte/macrophage lineage. It has also been detected in some sub-populations of B-lymphocytes (Maly et al. 1988), EBV-immortalised B cells (Maly et al. 1988; Maly et al. 1990) glomerular mesangial cells (Radeke et al. 1991), and several myelo-monocytic haematopoietic cell lines, including HL60 (Roberts et al. 1982) and U937 (Garcia et al. 1986). Recently, expression of the flavocytochrome b_{558} on pulmonary neuroepithelial cells has been associated with an O_2-sensitive potassium channel, and an oxygen sensing mechanism (Youngson et al. 1993).

HL-60 cells were derived from a patient with acute myeloblastic leukaemia with maturation (AML-M2), not promyelocytic leukaemia as originally described (Dalton et al. 1988). The cell line consists predominantly of promyelocytes, but has a small number of more mature cells including band cells and granulocytic cells. HL-60 cells can be induced to differentiate towards neutrophils when treated with DMSO, butyric acid, or retinoic acid (Koeffler, 1985). Induction with sodium butyrate and Mo cell-conditioned medium leads to basophilic differentiation (Hutt-Taylor et al. 1988). Under alkaline conditions, cells with atypical eosinophilic granules appear (Metcalf, 1983). Several agents induce monocytic differentiation, including phorbol ester (PMA), TNF-α, IFN-γ, and 1,25-dihydroxyvitamin D_3, and is associated with the appearance of pseudopodia, cribriform nuclei lacking nucleoli, and increased adherence to plastic.

Induced differentiation of these cells to a mature phenotype is generally associated with increased expression of both flavocytochrome b_{558} and cytosolic components of the NADPH-oxidase, and a parallel increase in activity up to six days post induction (Levy et al. 1990). IFN-γ is exceptional in that induction of differentiation occurs without cell cycle arrest (Roberts et al. 1989). Uninduced cells express low levels of immunoreactive $p47^{phox}$, and low levels of flavocytochrome b_{558}, yet membranes from these cells are able to support up to 40% of the superoxide produced by equivalent amounts of neutrophil membrane in cell-free assays (Levy et al. 1990). This suggests that significant amounts of flavocytochrome b_{558} are metabolically inactive, or that other membrane components are limiting. In contrast, immunoreactive $p67^{phox}$ protein induces later than the other components, and is the limiting factor for expression of complete cytosolic activity. As a consequence, uninduced cytosol does not support significant superoxide production. Monocytic differentiation and NADPH-oxidase activity can also be demonstrated using U937, a monocytoid cell-line derived from the pleural effusion of a patient with diffuse histiocytic lymphoma, which can be induced by agents such as retinoic acid, 1,25-dihydroxyvitamin D_3, and IFN-γ (Harris and Ralph, 1985).

Although non-phagocytic, EBV-immortalised B cells produce superoxide following stimulation with phorbol ester, or receptor dependent factors such as TNF-α, IL-1β and LPS (Hancock et al. 1990). Furthermore, these cells express all components of the NADPH-oxidase, and fail to produce superoxide if derived from patients with CGD (Volkman et al. 1984). A physiological function for the NADPH-oxidase in these cells is undetermined, but they have become useful models for investigation of NADPH-oxidase activity in whole cells. B cells derived from normal individuals only produce between 1 and 5% of the activity of mature neutrophils (Volkman et al. 1984), although there is considerable heterogeneity and is probably dependent on the maturation status of the cell. In contrast, cytosolic extracts from these cells have been shown to support the generation of high levels of superoxide in cell-free systems using neutrophil membranes (Cohen-Tanugi et al. 1991), suggesting that the comparatively low NADPH-oxidase activity of these cells relates to a deficiency of membrane components.

More recently, the X-linked genetic locus encoding the large subunit of the flavocytochrome was disrupted by homologous recombination in a PLB-985 XO human myelomonoblastic leukaemia cell-line, generating an *in vitro* model for X-linked CGD. These cells are bipotential and can be differentiated towards either granulocytic or monocytic forms which are both capable of NADPH-oxidase activity. Interestingly, only moderate levels of reconstituted expression of $gp91^{phox}$ are required to achieve NADPH-oxidase activity equivalent to that of the parent cell-line (Zhen et al. 1993).

1.4.2 Products of electron transport

Within the phagocytic vacuole of the neutrophil, as a result of electron transport through the membrane, one molecule of oxygen is reduced to a single molecule of superoxide anion (Babior et al. 1973). Potentially deleterious cytosolic acidification is prevented by the simultaneous activation of a homeostatic hydrogen ion extrusion mechanism, including a unique hydrogen ion conductance which is dependent on normal assembly of oxidase components (Qu et al. 1994). Interaction between two molecules of superoxide in a dismutation reaction results in the formation of oxygen O_2 and peroxide O_2^- (Del Maestro et al. 1980). Spontaneous dismutation in the absence of superoxide dismutase (SOD) occurs optimally at pH 4.8, but is rapid at physiological pH, and is followed by protonation of peroxide to form hydrogen peroxide. Although both H_2O_2 and O_2^- are produced in large amounts by stimulated phagocytes, and concentrations reach the millimolar range in the phagocytic vacuole, the ability of these molecules to react directly with biological materials is probably limited (Weiss, 1989). The generation of other more reactive oxygen free radicals *in vivo*, such as the hydroxyl radical (HO·) or singlet oxygen ($^1O_2$), and more recently of radicals derived from nitric oxide remains speculative (Klebanoff, 1988; Lowenstein and Snyder, 1992). In particular, these processes are dependent on the presence of free metals such as copper and iron, which may themselves be sequestered by granule proteins such as lactoferrin, and therefore unavailable for reaction.

1.4.3 Antimicrobicidal activity

In vitro, neutrophils from patients with CGD have markedly impaired microbicidal activity against specific micro-organisms, yet retain the ability to efficiently kill many others. Normal neutrophils incubated under anaerobic conditions exhibit impaired microbicidal activity against a similar restricted range of bacteria (Odell and Segal, 1991).

Myeloperoxidase, present in abundance in the primary granules of neutrophils and responsible for the yellow-green colour of pus, is capable of utilising H_2O_2 as a substrate to catalyse the oxidation of halide ions to hypohalous acids. In the neutrophil, hypochlorous acid is the predominant reaction product, and may interact with nitrogen containing compounds to form reactive and potentially microbicidal chloramine species. However, the halogenation reaction is not necessarily the major or natural function of myeloperoxidase, which may be more important as a scavenging molecule. Myeloperoxidase deficiency occurs with a prevalence of 1 in 2000 in the population, yet only six patients have been reported in the literature to have serious infections (Selsted et al. 1993). Four of these had disseminated or visceral candidiasis, and three of the four had concomitant diabetes mellitus. Furthermore, chicken neutrophils lack this enzyme. However, myeloperoxidase has been shown to reconstitute microbicidal activity of cytoplasts (neutrophils from which the granules and nuclei are removed, but which are able to mount a respiratory burst) when introduced into the phagocytic vacuole (Odell and Segal, 1988).

The NADPH-oxidase may primarily influence microbial killing by other mechanisms. An important consequence of failure of the NADPH-oxidase is an unusually rapid and extensive fall in pH within the phagocytic vacuole (Segal et al. 1981; Cech and Lehrer, 1984). In a normal phagocytic vacuole, activation of electron transport, and generation of H_2O_2 results in a dramatic consumption of protons, and a rise in pH to 7.8-8.0, before this slowly falls back to neutral levels. This is important because many of the proteolytic enzymes released into the vacuole are maintained in the granules at a pH of about 5.0, but have a neutral pH optimum. Under normal circumstances their release into the vacuole would coincide with an abrupt elevation in pH and killing and

digestion of the microbe. In CGD or anaerobic cells, this initial alkalinisation does not occur, and the excessively acidic environment is associated with inefficient enzymatic killing of microbes and retention of undigested cellular debris.

It is interesting to speculate that the NADPH-oxidase is in some way involved in processing of antigen. Phagocytosis of bacteria without a mechanism for entry into the cytosol, results in presentation of bacterial antigens by class I MHC molecules (Pfeifer et al. 1993). This process is resistant to classical inhibitors of the class I processing pathway, and suggests a novel pathway for processing of exogenous phagocytic antigens which may be partially dependent on NADPH-oxidase activity. Altered antigen presentation by CGD monocytes has been previously reported (Heijnen et al. 1986). More recently, a significant decrease in numbers of CD4+CD29+ and CD8+CD11b+ T-cells in 17 patients with CGD has been reported, and was suggested to reflect abnormal maturation of T-lymphocytes (Hasui et al. 1993).

1.5 MOLECULAR PATHOLOGY

The genes encoding both subunits of the flavocytochrome b_{558}, and four cytosolic factors, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and p21*rac*, have been cloned, and molecular lesions resulting in CGD identified in all but p21*rac* and $p40^{phox}$ (Royer Pokora et al. 1986; Volpp et al. 1989; Dinauer et al. 1990; Leto et al. 1990). The genetic loci for the first four components have been mapped to specific chromosomal locations. As predicted from the sex distribution of patients, the gene encoding the β-subunit of the flavocytochrome b_{558} maps to the X-chromosome, while the other components are autosomal (Table 1.1).

Large studies from the United States and Europe have identified the distribution of genetic lesions within CGD patients (Clark et al. 1989; Casimir et al. 1992). The European study determined the affected oxidase component in 63 CGD patients from 56 families, by Western blot analysis of neutrophil extracts. Overall, two thirds of families were found to have an X-linked defect of the β-subunit of the flavocytochrome b_{558}. The remaining patients had autosomal disease, with those exhibiting deficiency of $p47^{phox}$, the most prevalent. Defects in the small α-subunit of the flavocytochrome b_{558}, and in

$p67^{phox}$ made up the final 10% of cases. A lesion affecting the flavocytochrome b_{558} without evidence for an X-linked inheritance pattern may arise from A22 CGD or spontaneous mutation in the gene coding for the flavocytochrome b_{558} β-chain. In the European study, the established cases of A22 CGD were either female, or siblings of female patients, so that the true incidence may be underestimated. The observed genetic distribution of CGD is in close agreement with a similar study of 82 American families, although in this group, the incidence of A47 CGD was higher (Clark et al. 1989).

1.5.1 X-linked, flavocytochrome b_{558}-negative CGD

$gp91^{phox}$ is expressed almost exclusively in terminally differentiating myelomonocytic cells, and was the first human gene to be cloned based on the knowledge of its chromosomal location (Royer Pokora et al. 1986). A patient with a large deletion of the X-chromosome and four genetic disorders, Duchenne muscular dystrophy, retinitis pigmentosa, McCleod syndrome, and CGD, facilitated the isolation of gene sequences specific to this region. The library constructed from these sequences was screened with cDNA specific to induced HL-60 cells, and fragments were isolated that mapped to the deleted region in the CGD patient. One identified clone hybridised to a myeloid specific mRNA that was found to be abnormal in abundance or structure in four patients with X-linked CGD. Surprisingly, amino acid sequence from purified cytochrome b_{558} did not correlate with predicted sequences from the cloned gene, and it was not until the flavocytochrome b_{558} was found to consist of two subunits, and partial amino acid sequence was obtained from the β-subunit that the identity of the cloned gene was confirmed (Dinauer et al. 1987; Teahan et al. 1987).

Molecular lesions at the corresponding genetic locus, CYBB, account for the majority of cases of CGD. The mutations are particularly heterogeneous, and are unique to individual families in over 90% of cases described (Curnutte, 1993; Roos et al. 1993). The most frequently encountered lesions are missense mutations resulting in non-conservative amino acid substitutions, deletions in genomic DNA which may be restricted to the gene locus or involve larger regions of the X chromosome, and mutations resulting in RNA splicing defects. As expected, missense mutations result in considerable heterogeneity of biochemical phenotype.

Table 1.1: Characteristics and distribution of gene defects in CGD.

Component	gp91^{phox}	p22^{phox}	p47^{phox}	p67^{phox}	p40^{phox}	p21rac
Disease	X-linked X91 CGD*	Autosomal recessive A22 CGD*	Autosomal recessive A47 CGD*	Autosomal recessive A67 CGD*	Not known	Not known
Results from European study of 56 families: *Numbers of affected families, and incidence.*	X91^0 35(63%) X91$^-$ 2(4%)	A22^0 3(5%)	A47^0 13(23%)	A67^0 3(5%)	Possibility of p40^{phox} molecular lesion in some 'A67 CGD'	
Genetic locus	CYBB	CYBA	NCF-1	NCF-2		
Chromosomal location	Xp21.1	16q24	7q11.23	1q25		
Gene/mRNA size	30kb/4.7kb	8.5kb/ 0.8kb	17-18kb/ 1.4kb	37kb/ 2.4kb	1.2kb	rac1/ 2.4+1.1kb rac2/ 1.5kb
Exons	13	6	9	16	Not known	Not known
Tissue specificity	myeloid Low levels in mesangial cells, and some B lymphocytes. Pulmonary neuroepithelial bodies.	mRNA ubiquitous Protein expression only in presence of gp91^{phox}	myeloid	myeloid	myeloid	p21rac1, widespread p21rac2, myeloid

* Accepted classification of CGD, in which A or X denote inheritance pattern This is followed by the molecular weight of the affected component in kDa. The superscript refers to the level of detectable immunoreactive protein: (0) indicates no protein, (-) indicates diminished protein, and (+) indicates normal levels of defective protein.

An interesting group of splicing defects arise from nucleotide substitutions at both donor and acceptor splice sites (de Boer et al. 1992a). These usually result in deletion of complete exons, although in one patient a missense mutation in exon 6 created a new donor splice site that was used in preference to the usual site. In another, mutation at the splice acceptor site of exon 12 resulted in use of a cryptic site 30 nucleotides into exon 12. As a result, 30 nucleotides are spliced out of the mature gp91^{phox} transcript (Schapiro et al. 1991). Premature stop codons as a result of nucleotide insertions, or missense mutations, invariably lead to undetectable levels of flavocytochrome b_{558}. Recently, two mutations between the CCAAT and TATA box motifs of gene promoter have been shown by gel-shift assay to be associated with two variant forms of CGD, in which levels of immunoreactive protein and NADPH-oxidase activity are decreased (Newburger et al. 1994).

1.5.2 Autosomal recessive, flavocytochrome b_{558}-negative CGD

The gene structure, chromosomal location, and identity of mutations in flavocytochrome b_{558}-negative autosomal recessive CGD were first described in 1990 (Dinauer et al. 1990). Unlike other components of the NADPH-oxidase, the transcript for p22^{phox} is found ubiquitously, and in relatively primitive myeloid cells (Levy et al. 1990). Protein expression is however restricted to cells in which gp91^{phox} is also expressed. Molecular lesions are again heterogeneous and have included both homozygous and missense compound heterozygous mutations, DNA deletions, and one homozygous donor splice site mutation resulting in deletion of exon 4 (Dinauer et al. 1991; de Boer et al. 1992b; Roos et al. 1993). One mutation in the cytosolic domain, P156Q, which diminishes association of cytosolic components with physically intact flavocytochrome has been described earlier (Leusen et al. 1994).

1.5.3 Autosomal recessive, cytochrome b_{558}-positive CGD

The second most common cause of CGD is A47^{0} CGD. The p47^{phox} gene is expressed exclusively in terminally differentiating myelo-monocytic cells, and to a lesser extent in some sub-groups of lymphoid cells (Rodaway et al. 1990). EBV-immortalised B-cells express levels of protein similar to that of myeloid cells (Cohen-Tanugi et al. 1991). In

contrast to X91 CGD, a single lesion in the $p47^{phox}$ gene accounts for over 90% of mutant alleles. A GT dinucleotide deletion at a GTGT repeat at the boundary between the first intron and second exon results in a chain terminator at amino acid residue 51 (Casimir et al. 1991). Whether these are founder mutations or represent frequently occurring new mutations is unknown, but in view of the relatively high frequency of carrier status (1 in 2000 alleles), it is interesting to speculate on a survival advantage for the whole organism. Perhaps phagosomal acidification is protective against mycobacterial infection (Sturgill-Koszycki et al. 1994). A few compound heterozygotes with a missense mutation in combination with a GT deletion have also been described (Chanock et al. 1991).

The gene structure for $p67^{phox}$ has been described, but little is known about its regulation, or about mutations arising in A67 CGD (Kenney et al. 1990; Kenney et al. 1992). One mutation has recently been described which predicts a Gly-78 to Glu replacement (de Boer et al. 1994).

1.5.4 Molecular diagnosis of CGD

Immunoblotting for individual components of the NADPH-oxidase identifies the defective protein in the majority of cases. However, molecular lesions in either component of flavocytochrome b_{558} usually results in absence of the entire heterodimer. Although the site of mutation may be inferred from inheritance, it remains difficult to distinguish between males who may have acquired a new mutation at CYBB from the maternal germline, and those with autosomal recessive deficiency of $p22^{phox}$. Identification of the defective component may also be difficult in rare individuals who express relatively normal levels of protein which is functionally deficient. In these cases, complementation studies in a cell-free system using recombinant protein, or in whole cells by monocyte fusion, may be helpful (Roos et al. 1992; Curnutte, 1993).

Prenatal diagnosis can be made by evaluation of neutrophil function in foetal blood samples obtained at 14-16 weeks gestation (Levinsky et al. 1983; Newburger et al. 1979). Increasingly, genetic analysis of foetal tissue obtained from chorionic villous sampling, or cultured amniotic fibroblasts will replace functional assay on cord blood

for families at high risk, and obviates the need for second trimester termination. This strategy is dependent on identification of specific family based mutations or on informative polymorphisms. To date, one informative polymorphism has been identified within NCF2, and two within CYBB which are informative in about 50% of families (Bhattat and Franke, 1989; Muhlebach et al. 1990; Pelham et al. 1990; Kenney et al. 1991). Polymorphism identified at three identified tandem repeat sequences within CYBB may further improve the chances of tracking the mutant allele (Gorlin, 1991). Positive diagnosis may also be made by identification of a family-based mutation. As an alternative to genetic analysis, it may be possible to detect the presence of individual NADPH-oxidase components in chorion-derived macrophages with specific antibody (Nakamura et al. 1990).

1.6 CLINICAL FEATURES

1.6.1 The pattern of clinical disease

Chronic granulomatous disease (CGD) is a heterogeneous group of disorders characterised biochemically by disordered function of the NADPH-oxidase of phagocytic cells. It is estimated to affect between 1 in 500,000 and 1 in 1,000,000 individuals, although it is probably underdiagnosed because of a general lack of awareness of the condition, and because some individuals exhibit a mild clinical phenotype. Clinically, it is characterised by recurrent bacterial and fungal infections which are relatively resistant to treatment by conventional means. The majority of patients are now diagnosed before their second birthday, although some remain undiagnosed until later childhood, or even adult life (Finn et al. 1990). Phenotypic heterogeneity amongst patients with CGD is understandable in the light of the diverse molecular pathology, but even those with identical genetic defects may exhibit quite different clinical patterns. The eldest recorded patient with CGD presented with his first serious infection at the age of 69 years (Schapiro et al. 1991). In contrast, a grandson died at the age of five years from *Pseudomonas cepacia* pneumonia. An often stated dogma is that patients with X-linked CGD manifest the most severe clinical phenotype (Weening et al. 1985), and although this is probably true, it should not obscure the fact that some patients with autosomal inheritance patterns can be equally severely affected.

Interestingly, and somewhat surprisingly, 'variant' patients, so-called because they retain partial activity of the NADPH-oxidase, may present with manifestations of classical disease no different from those with undetectable activity.

Infections usually predominate at epithelial surfaces in direct contact with the environment, in particular the skin, mucous membranes, lung and gut (Hayakawa et al. 1985; Forrest et al. 1988; Mouy et al. 1989; Finn et al. 1990). This is reflected in the most common presenting features; lymphadenitis, cutaneous infection and dermatitis, pulmonary infection, persistent fever, and diarrhoea. Deep seated infection such as osteomyelitis, visceral abscess formation, particularly in the liver, and septicaemia are frequently encountered, and failure to thrive is a common response to chronic infection in childhood. Pulmonary infection with aspergillus is not uncommon, is particularly difficult to eradicate, and is associated with a high mortality rate in spite of appropriate treatment. Gastrointestinal manifestations are varied, and often result in misdiagnosis and implementation of inappropriate treatment. These include diarrhoea, often as a result of *Salmonella* infection, recurrent perianal abscess formation, and extensive granulomatous enteritis which may clinically, and radiologically, indistinguishable from Crohn's disease, and which may likewise be complicated by lumenal obstruction (Ament and Ochs, 1973). Obstructive lesions are not restricted to the lower gut, and have been associated with narrowing of the oesophagus, gastric antrum, and ureteric orifices as a result of granulomatous cystitis. Hepatomegaly, splenomegaly, and dermatitis are other manifestations of the characteristic granulomatous reaction.

1.6.2 Pathological features of disease

The microbial organisms responsible for the majority of infections are characteristic of this disorder; *Staphylococcus aureus* and enteric Gram-negative rods such as *Salmonella*, *Klebsiella*, *Aerobacter*, *Serratia* and *Pseudomonas* account for most bacterial pathogens, whilst *Aspergillus* species are the most commonly implicated fungi. Although numerous other pathogens have been described in association with CGD, the most striking feature of the common pathogens is that they produce a natural scavenging enzyme, catalase, which neutralises H_2O_2. Catalase-negative organisms such as *Streptococci* do not appear to present particular problems. The most commonly cited

explanation for this observation is that small amounts of H_2O_2 are generated by the micro-organisms themselves within the phagocytic vacuole. This may be sufficient to activate some cellular microbicidal activity in the absence of a functional NADPH-oxidase, but is effectively neutralised by catalase. Frequently the pathogenic organism is not identified, and treatment remains empirical.

The characteristic histopathology is widespread granulomatous infiltration of tissues, probably as a result of inefficient cellular attempts to eliminate infectious agents, and digest unwanted cellular debris. The granulomata are composed of numerous giant cells and lipid laden histiocytes. The high levels of cytokines produced by, and maintaining this inflammatory response, contribute to the marked cachexia which is seen in some patients, and may be amenable to therapeutic manipulation. Patients are frequently anaemic, usually as a consequence of chronic disease, but sometimes as a result of malabsorption syndromes, for example of vitamin B_{12}, secondary to enteric disease. They are characteristically hypergammaglobulinaemic, with a raised erythrocyte sedimentation rate, even when apparently uninfected, and develop a leukocytosis during septic episodes.

1.6.3 Prognosis

Published retrospective studies suggest a survival rate at 10 years of between 50 and 70%, although from the age of 20 years onwards, survival is maintained at about 50% (Mouy et al. 1989; Finn et al. 1990). The introduction of prophylactic therapy over the past few years, together with availability of more powerful broad spectrum antibiotics has undoubtedly resulted in a greatly improved outlook, and a reduction in associated morbidity and mortality.

1.6.4 Functional Diagnosis of Chronic Granulomatous Disease

The diagnosis of CGD is confirmed by demonstration of absent or markedly deficient respiratory burst activity in phagocytic cells. Other aspects of phagocyte function including chemotaxis, adhesion, phagocytosis and degranulation are normal. The simplest and most widely available screening test for CGD is the NBT (nitroblue tetrazolium) microscope slide test (Ochs and Igo, 1973; Segal, 1974; Weening et al.

1974). This qualitative test assesses the ability of an activated cell to produce superoxide, the primary product of the NADPH-oxidase, by reduction of the yellow water soluble NBT dye, to insoluble blue formazan, which precipitates on the activated cell. This test has the advantage of being able to detect the carrier state in female relatives of patients with X-linked CGD, in which cases there are a mixed population of NBT-positive and NBT-negative cells. Occasionally, preferential inactivation of the abnormal X-chromosome in carrier females results in an apparently normal NBT test, while preferential inactivation of the normal chromosome may result in a CGD phenotype. Carriers of autosomal types of CGD show little abberation of oxidase function, although reduced production of superoxide by neutrophils obtained from carriers of $A47^0$ and $A67^0$ CGD has been described (Verhoeven et al. 1988; de Boer et al. 1994). Detection is best achieved by genetic analysis. Variant CGD patients, so called because of their ability to produce small amounts of superoxide, sometimes up to 30% of normal, and patients with severe glucose-6-phosphate dehydrogenase deficiency (in which cellular replenishment of NADPH is deficient) may produce a negative NBT test .

Quantitation of the respiratory burst is important to confirm the diagnosis in the case of an abnormal screening test, or in cases where the diagnosis is in doubt. This can be done either by direct measurement of oxygen consumption with an oxygen electrode, or by measuring the products of electron transport. The most widely used measure of superoxide production is the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c (Weening et al. 1975). Chemiluminescent assays, such as SOD-inhibitable reduction of lucigenin, or catalysed peroxidation of luminol, are less quantitative, but nonetheless are more specific indicators of NADPH-oxidase activity (Wymann et al. 1987; Porter et al. 1992), and are much more sensitive. Activation *in vitro* is commonly produced by soluble stimuli, such as phorbol myristate acetate (PMA) and formyl-methionyl-leucyl-phenylalanine (fMLP), or particulate stimuli, such as opsonised bacteria or zymosan.

1.7 CLINICAL MANAGEMENT OF CGD

The most important principles of treatment in this primary immunodeficiency syndrome are of prevention and aggressive treatment of infection (Fischer et al. 1993).

1.7.1 General measures

Patients with CGD are not overtly susceptible to viral infection, and routine childhood immunisation against mumps, rubella and measles is not contra-indicated. Similarly, immunisation with toxoids, or capsular polysaccharide (as in Hib vaccines) can be given routinely. However, vaccines against bacteria in which the immunogen is live albeit attenuated, for example BCG and the recently available oral typhoid vaccine, should be avoided except under exceptional circumstances. Skin wounds should be treated with topical antiseptic agents, and close attention be paid to dental hygiene, and to protection in particular of mucosal surfaces. Exposure to fungal spores in decaying plant material, and wood chippings should be avoided. Nutrition should be adequately maintained, and supplemented if necessary, particularly in the presence of a malabsorption syndrome. If oral intake remains inadequate, then consideration may be given to enteral, or even parenteral feeding. Anaemia of chronic disease responds well to subcutaneous erythropoietin in the presence of an adequate supply of haematinics, and is associated with improved sense of well-being, although it is not clear whether this influences the course of the disease. Unfortunately, some patients with X-linked CGD have the rare Kell blood phenotype Mcleod, which should be determined before blood transfusion is contemplated.

1.7.2 Prophylactic treatment

Although no prospective controlled trial has taken place, retrospective analyses of patient records from both the United States and Europe have shown that prophylactic antibiotics are effective in prolonging the period between major infections (Mouy et al. 1989; Margolis et al. 1990). In one study from the NIH, prophylactic administration of trimethoprim-sulphamethoxazole to a group of 36 patients resulted in a decrease of non fungal infection from 7.1 to 2.4 per 100 patient months in patients with autosomal CGD, and from 15.8 to 6.9 infections per 100 patient months in patients with X-linked

inheritance. There is as yet no evidence that the frequency of fungal infection, in particular by *Aspergillus*, is reduced by prophylactic administration of antifungal agents, although itraconazole has shown promise in retrospective studies of neutropenic patients, and is currently undergoing evaluation in CGD (van't Wout, 1992). Other antibiotics have not been formally evaluated.

Interferon-γ (IFN-γ) is an immunomodulatory cytokine secreted predominantly by TH (T-helper) cells following antigen-specific activation. It enhances MHC class II expression on antigen presenting cells, and contributes to late differentiation of B-cells. It also acts as a potent macrophage activating factor, and synergizes with tumour necrosis factor (TNF) to enhance susceptibility of target cells to T-cytotoxic (TC) and natural killer (NK) cells (Todd and Goa, 1992). On the basis of *in vitro* studies in which IFN-γ resulted in partial restoration of NADPH-oxidase activity in neutrophils and monocytes from selected patients with X-linked CGD, four patients were treated with 2 consecutive subcutaneous injections of IFN-γ (Ezekowitz et al. 1987; Ezekowitz et al. 1988). Superoxide production increased in all four patients, peaking after 1-2 weeks in monocytes, and 2-3 weeks in granulocytes. This correlated with enhanced microbicidal activity 2 weeks after treatment, and some increase in levels of immunoreactive cytochrome b. However, the delayed peak of superoxide production after 2 consecutive doses of IFN-γ is not consistent with the circulating half life of neutrophils, which can be measured in hours, and it has been suggested that IFN-γ acts on progenitor cells, rather than their differentiated progeny (Ezekowitz, 1992). These studies on selected patients with X-linked CGD, together with *in vitro* evidence for increased microbicidal activity of IFN-γ treated cells in the absence of enhanced respiratory burst activity, prompted a Phase III multicentre clinical trial (Gallin, 1991). In this double-blind, placebo controlled study 128 patients with CGD were randomised to receive placebo or IFN-γ at a dosage of 50g per m^2 , administered subcutaneously three times per week. The primary end-point was the length of time before serious infection requiring hospitalisation and treatment with parenteral antibiotics. The trial was prematurely terminated after 9 months, at which point IFN-γ treated patients had experienced a 67% reduction in relative risk of serious infection compared to the placebo group, and had spent two thirds less time in hospital. Children aged less than 10 years benefited most

from treatment, which was generally well tolerated. However, the frequency of infection in patients from some European centres receiving antibiotic prophylaxis alone, was less than in the IFN-γ treated trial group as a whole (Mouy et al. 1991). Furthermore, IFN-γ did not reduce the rate of serious infections in the European patients who participated in the trial. Although the number of participating European patients does not permit statistical evaluation of this observation, the explanation for this difference is not clear, but seems unlikely to arise from differences in patient groups.

In contrast to earlier studies, participants in the multicentre study and other groups have been unable to demonstrate restoration of neutrophil NADPH-oxidase activity, or increased expression of individual oxidase components (Muhlebach et al. 1992; Woodman et al. 1992). The mechanism of action of IFN-γ in the majority of patients with CGD is therefore speculative, but almost certainly reflects an influence on aspects of immunity divorced from the NADPH-oxidase. In spite of this, IFN-γ appears to represent a useful therapeutic tool in CGD, and perhaps should be administered prophylactically to identified patients at high risk of infection. The value of IFN-γ when used to augment conventional treatment during septic episodes, is unknown, although there have been numerous anecdotal reports.

Some groups have investigated the ability of other cytokines to modulate the respiratory burst in cells derived from CGD patients, including one which reported enhanced superoxide production by CGD monocytes cultured in the presence of IFN-γ, TNF, IL-3 and IL-1 (Jendrossek et al. 1993). Recombinant GM-CSF has been tested in a variant CGD patient with a hepatic abscess, but despite a profound increase in the number of circulating leukocytes, there was no detectable improvement in oxidase function, or clinical condition, during the period of administration of the cytokine (Muhlebach et al. 1991). Recombinant G-CSF has been used sporadically in septic CGD patients, but it is impossible to isolate any potential benefit over conventional treatments.

1.7.3 Treatment of active infection

Conventional treatment for intervening sepsis in CGD is focused on aggressive antimicrobial chemotherapy, initially directed at characteristic pathogens. In many cases, the pathogen is not isolated and treatment remains empirical. Modern drugs such as ceftazidime and ciprofloxacin, which both have anti-pseudomonal activity, in combination with teicoplanin which has broad spectrum activity against Gram positive organisms, have proved to be useful in resistant cases, particularly in the absence of an identified pathogen. Intravenous amphoteracin remains the mainstay of treatment for aspergillus infection, although itraconazole, which is only available as an oral preparation, may be more effective, and better tolerated. Corticosteroids carry an additional risk of immunosuppression, but may be useful in situations where an intense granulomatous reaction has resulted in stricture formation and lumenal obstruction (Chin et al. 1987). Florid dermatitis may also respond to topical steroids, but must be used in combination with appropriate antibiotics, and topical antifungal agents. Neutrophils obtained from leukophoresed donors may be used as an adjunct to therapy, and can be given intravenously, or even directly applied to the site of infection. Transfused neutrophils have been demonstrated in bronchoalveolar lavage fluids for up to 24 hours, and transfused monocytes may persist for longer periods (Buescher and Gallin, 1982). Pre-treatment of donors with rhG-CSF can be used to increase the yield. However, difficulty with purification and storage of adequate numbers of cells, potential transmission of viruses, and the possibility of graft-versus-host disease (GvHD) and the formation of allotypic antibodies limit use of transfused allogeneic neutrophils to cases resistant to conventional treatment.

1.8 CURATIVE THERAPY: BONE MARROW TRANSPLANTATION

1.8.1 Application and complications of bone marrow transplantation

The haematological consequences following irradiation of survivors of the Hiroshima and Nagasaki atomic bombs stimulated considerable research into the potential of bone marrow to confer radioprotection to experimental animals. It was later shown that mice would recover from lethal irradiation if haematopoietic areas in the long bones were shielded, and that spleen cell, or bone marrow transfusions were protective (Lorenz et

al. 1952). These observations provided a means to correct bone marrow failure syndromes, and to protect patients against the myeloablative effects of radiation and chemotherapy. However, early attempts at allogeneic bone marrow transplantation (BMT) failed as a result of immunological graft rejection and 'secondary disease' (later characterised as GvHD), and it wasn't until the discovery of the human leukocyte antigen (HLA) system that successful transplantation became possible.

The first successful HLA matched sibling transplants were performed in two distinct primary immunodeficiency disorders, severe combined immunodeficiency (SCID), and Wiscott-Aldrich syndrome (Bach et al. 1968; Gatti et al. 1968). SCID is a heterogeneous group of disorders characterised by profound reduction or absence of T lymphocytes. Uniquely, sibling matched transplants in these conditions are effective in the absence of additional immunosuppressive conditioning (which otherwise is required to achieve consistent engraftment), and cure rate approaches 90% (Fischer et al. 1990). T lymphocyte depleted haploidentical parental transplants are curative in over 70% of patients.

Transplantation of immunodeficiencies other than SCID is complicated by the need for additional conditioning immunosuppression usually achieved by a combination of cyclophosphamide and either the alkylating agent busulphan, or whole body irradiation (Morgan, 1992). Total mortality following a fully matched (identical at all 6 HLA and on average 50% minor histocompatability loci) sibling graft for inherited immunodeficiencies excluding SCID, and in which conditioning is necessary for engraftment, has been estimated to be about 30%. Morbidity and mortality relate primarily to the high degree of immunosuppression required to achieve engraftment, and to GvHD. GvHD is a complex and incompletely understood syndrome largely induced by recognition of major histocompatability complex (MHC) Class I or II antigen differences in the host, and clonal expansion of corresponding donor lymphocytes (Barrett, 1992). Histologically, cell damage is restricted to proliferating cells of the cutaneous epithelium and gastrointestinal tract, and proliferating cells of the bone marrow and lymphoid system. Even with modern matching techniques and adequate prophylaxis with cytotoxic agents and cyclosporin, GvHD remains one of the

major complications of BMT, and occurs in up to 25% of patients recieving fully MHC-matched sibling grafts. Prevention by T cell depletion of donor cells is extremely effective, but is associated with markedly increased rates of graft rejection (Poynton, 1992). Directly or indirectly GvHD is responsible for 25% of post transplant mortality.

For the reasons outlined above, allogeneic transplantation for CGD has been successful in a only a small number of patients (Table 1.2), and the risks associated with the procedure outweigh potential benefits in the majority of cases. Patients with MHC-identical siblings who fail to respond to optimal conventional treatment are probably the best candidates if not chronically infected, but use of matched unrelated or non-identical family donors is inadvisable. An alternative strategy which may eventually overcome the problems of allogeneic transplantation, and which is not limited by supply of suitable donor material, is somatic gene therapy.

Table 1.2 Published BMT experience for CGD

(Seger and Ezekowitz, 1994)

Year Reported	Patient age and sex	Donor	Period of engraftment Chimerism (%)	Outcome
1976	Male / 3yr	Compatible sister	2mo (100%)	Rejection
1977	Male / 2yr	MUD	7yr (12%)	Rejection
1977	Male /7yr	MUD	3mo (33%)	Rejection
1982	Male /15yr	Compatible sister	4mo (100%)	cGvHD*
1984	Male /5mo	Compatible brother	6yr+ (23%)	Clinical cure
1989	Male /11yr	Compatible sister	3yr+ (100%)	Clinical cure
1991	Male /7yr	Compatible sister	2yr+ (100%)	Clinical cure
1992	Male /8mo	MUD	3yr+ (100%)	Clinical cure

MUD= matched unrelated donor * death as complication of transplant

1.9 SOMATIC GENE THERAPY

Recent developments in understanding of the molecular basis of both inherited and acquired disease have stimulated new treatment strategies based on functional gene transfer, or direct inhibition of gene expression. Somatic gene therapy can be defined most simply as the introduction of genetic material into somatic cells in order to treat or modify disease. Application of this technology was first proposed for curative therapy of classical inherited genetic disease, in which complementation of a cellular deficiency was envisaged to be the most realisable target, but attention and resources have now extended to oncological disease, HIV-related acquired immunodeficiency, and even complex multigenic disease in which the objective is to provide supplemental therapeutic or pharmacological benefit. Although target diseases for somatic gene therapy are now large in number and varied in aetiology, the technologies that are required for successful gene transfer and expression are common to many. Depending on the disease process, and the nature of the new genetic material, cellular function may be modified in several ways.

Mechanism of therapeutic action

- Complementation of inherited genetic defect
- Expression of new therapeutic protein or immunogen
- Expression and secretion of systemic therapeutic agent
- Inhibition of abnormal cellular activity
- Inhibition of viral replication

1.8.1 Somatic gene therapy for inherited immunodeficiency

As the molecular basis for some of the primary immunodeficiencies has become established, they have become candidates for application of gene therapy (Cournoyer and Caskey, 1993). The most widely explored disease is adenosine deaminase (ADA) deficiency, which is responsible for 15-25% of all cases of SCID, and although a rare disorder, is in many ways an ideal model for development of haematopoietic gene therapy protocols.

Ideal candidate disorder for application of somatic gene therapy

- Correctable by allogeneic BMT
- Molecular genetics characterised
- Function of gene product characterised
- Constitutive gene expression
- Selective advantage for corrected cells
- Severe disease phenotype
- Alternative therapy inadequate or unavailable

As an alternative candidate disorder for somatic gene therapy, CGD fulfils many of these criteria, although restoration of biochemical function to CGD cells is unlikely to produce selective growth advantage. Fully regulated expression of oxidase components is preferable, but may not be necessary for correction of the biochemical phenotype.

1.8.2 Target cell populations for therapeutic gene transfer

The objective of therapeutic gene transfer for CGD is lifelong restoration of NADPH-oxidase function in peripheral blood phagocytes, particularly neutrophils and monocytes. In contrast to mature neutrophils which are short-lived transcriptionally quiescent cells, and therefore not appropriate targets for long term correction of disease phenotype, differentiated tissue macrophages survive for longer periods and may be a target for transitory corrective therapy (Buescher and Gallin, 1982). The optimal target cell population is the pluripotent haematopoietic stem cell (PHSC), defined by the capacity for extensive self-renewal, and retention of multilineage differentiation potential (Till and McCulloch, 1961; Dexter et al. 1977).

1.8.4 Gene transfer to eukaryotic cells

Non-viral methods of gene transfer were the first to demonstrate correction of a disease phenotype *in vitro* (Mulligan and Berg, 1981), and the first to be used albeit unsuccessfully, in a clinical setting. At the present time, the preferred technology, at least for haematopoietic cells, is virus-based. Studies on RNA and DNA tumour viruses in the late 1960's and early 1970's indicated that transformation of the cell was associated with permanent transfer of viral genetic material. The emergence of

recombinant DNA technology meant that viruses could be engineered to deliver heterologous genetic material to eukaryotic cells with much greater efficiency than was achievable using non-viral systems, and for some viruses resulted in stable integration of this new material into the host cell genome. The virus systems that have been most applied to haematopoietic cells are based on retroviruses, adeno-associated virus, and adenoviruses, which will be discussed in more detail later. Other gene delivery methods under development include those based on alternative viruses (Jolly, 1994), and those based on non-viral systems or hybrid systems which incorporate some viral functions (Michael and Curiel, 1994).

1.8.5 Somatic gene therapy for CGD

The following studies explore the feasibility of gene transfer to cells which display the biochemical phenotype typical of CGD, and investigate mechanisms which may enhance the efficiency of transfer, and which may be necessary for sustained and regulated gene expression.

1.9 AIMS OF THESIS

- *To investigate transcriptional regulation of the $p47^{phox}$ gene.*

- *To reconstitute NADPH-oxidase activity in cells derived from patients with $p47^{phox}$-deficient chronic granulomatous disease.*

- *To investigate gene transfer vector systems that will optimise transduction of pluripotent haematopoietic stem cells.*

CHAPTER 2:

MATERIALS AND METHODS

Unless otherwise indicated, all enzymes were supplied by Promega, and all chemicals by Sigma.

2.1 EXTRACTION AND PRECIPITATION OF DNA

2.1.1 Phenol extraction

Aqueous solutions containing nucleic acid, but contaminated with protein were de-proteinised by extraction with phenol. For extraction of plasmid and genomic DNA, an equal volume of 0.5M Tris-HCl (pH8.0) saturated phenol:chloroform:isoamyl alcohol (24:24:1), was thoroughly mixed with the solution containing nucleic acid, and centrifuged to separate organic and aqueous layers. DNA (and RNA) was precipitated from the aqueous phase. For heavily contaminated solutions, the extraction was repeated. For preparation of RNA, phenol was unbuffered, and RNA selectively recovered from the aqueous phase.

2.1.2 Precipitation of nucleic acid

Except where indicated, genomic and plasmid DNA were precipitated with 2-2.5 volumes of ethanol and 0.1 volumes of 3M sodium acetate pH5.5, or 0.1 volumes of 5M lithium chloride, which is more soluble in ethanol, at a temperature of 0 to -20^0C. RNA was precipitated with 2.5 volumes of ethanol in the presence of 0.1 volumes of 3M sodium acetate pH5.5. Oligonucleotides were precipitated with 2.5 volumes of ethanol in the presence of 0.1 volumes of 5M sodium chloride. Precipitation of RNA and oligonucleotides was completed overnight at -20^0C.

2.2 AMPLIFICATION OF PLASMID DNA IN BACTERIA

2.2.1 Preparation and transformation of competent cells

Competent bacterial cells (JM109 or XL1Blue strains, Stratagene) were prepared by a variation of the original method of treatment with ice-cold $CaCl_2$ (Mandel and Higa, 1970). Cells were grown overnight in LB at 37^0C with agitation. A 5ml inoculum of the overnight culture was grown in 130ml of the same media to a density defined by an absorbance at 600nm of 0.4-0.6. The culture was centrifuged for 5 minutes at 6000*g* (4^0C). Cells were resuspended in ice cold 50mM $CaCl_2$ at half original volume, and incubated on ice for 1 hour before being pelleted as before, and resuspended in 50mM $CaCl_2$ at 0.05 of the original volume. The cells were again left on ice for 1 hour. Freshly prepared cells were mixed with 15% glycerol, and frozen at -70^0C for later use. For transformation, competent cells were thawed on ice, and incubated with plasmid DNA for 20 minutes, before heat shock at 42^0C in a pre-warmed water bath for 90 seconds. LB (0.5mls) at room temperature was added to the mix, and incubated with gentle shaking at 37^0C for 1-2 hours. Bacteria were concentrated by centrifugation, resuspended in 200µl of the same media, and spread on 1.5% LB-agar plates (containing 50µg/ml ampicillin unless otherwise stated). Plates were incubated overnight at 37^0C to allow for growth of resistant colonies.

2.2.2 Amplification and recovery of recombinant plasmid DNA

Bacterial colonies from agar plates were innoculated into 10ml of media (LB or TB) containing antibiotics to which the recombinant plasmid conferred resistance (usually 50µg/ml ampicillin) and incubated at 37^0C with agitation for 12-36 hours. For bulk cultures, 5mls of culture was used to inoculate an additional 100-250mls of the same media, which was incubated as above. For efficient recovery of low copy number plasmids (particularly pBR322 derivatives) containing the ColE1 replicon, bacteria were grown to a density defined by an absorbance of 0.5-0.8 at 550nm, and supplemented with chloramphenicol (170µg/ml) to block protein synthesis. Incubation was continued overnight. Plasmid DNA was recovered by a modified alkaline lysis method. For large scale recovery, nucleic acid was recovered by alkaline lysis and precipitation with isopropanol. Resuspended plasmid DNA was further purified by precipitation of high

molecular weight RNA with 5M LiCl, and digestion of low molecular weight RNA with RNAase (20μg/ml). DNA and contaminating protein was precipitated with an equal volume of ice-cold polyethylene glycol (1.6M NaCl, 13% (w/v) PEG 8000). Plasmid DNA was finally deproteinised by extraction with phenol:chloroform:iosoamyl alcohol, and precipitated from the aqueous phase with salt and ethanol. For small scale preparations, nucleic acid was prepared from alkaline-lysed bacteria by elution from a silica-based resin ('Magic Minipreps', Promega), according to manufacturers instructions.

2.2.3 Preparation of genomic DNA from cells

High molecular weight DNA was prepared from cells by addition of 1ml of DNA lysis buffer (10mM Tris-HCl, pH8.0, 5mM EDTA, 0.5% (w/v) SDS, 150μg/ml proteinase K added just before use) per ~10^7 washed cells, and incubation overnight at 37^0C. The cell lysate was extracted twice with phenol, and DNA precipitated with ethanol.

2.2.4 Preparation of low molecular weight DNA from cells and virus supernatant

Low molecular weight DNA was prepared from cells or from tissue culture supernatant by a modified Hirt extraction (Hirt, 1967). Briefly, 50μl of 10 x buffer (100mM Tris-HCl, pH7.5, 6% SDS (w/v), 100mM EDTA, proteinase K 100μg/ml, yeast tRNA 50-100μg/ml) was added to 0.5mls of cell lysate or supernatant in an Eppendorf tube, and incubated at 37^0C for 2-3 hours. 110μl of 5M NaCl was added to precipitate high molecular weight DNA with SDS at 4^0C for 4-16 hours. The precipitate was pelleted at 13,000rpm in a microfuge, and low molecular weight DNA recovered from the aqueous phase by phenol extraction and ethanol precipitation.

2.2.5 Preparation of RNA from cells and tissue culture supernatant

RNA was prepared from washed cells using acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi, 1987) and stored in isopropanol at -70^oC. Where necessary, solutions were pre-treated with DEPC to inactivate RNAase. Before use, the sample was centrifuged at 12,000*g* for 15 minutes and the RNA pellet washed in 70% ethanol. Viral RNA was isolated from tissue culture supernatants by addition of 50μl of 10 x RNA extraction buffer (as for Hirt buffer, but constituents DEPC treated where possible) to 0.5mls of supernatant in an Eppendorf tube, which was incubated at 37^0C for 2-3 hours.

Viral RNA and carrier was recovered from the aqueous phase by phenol extraction and ethanol precipitation.

2.3 QUANTITATION OF NUCLEIC ACID

2.3.1 Ethidium bromide and spectrophotometric quantitation

The concentration of DNA was estimated by comparing 1µl of sample with 1µl dilutions of lambda (l) DNA (1-100ng/ml, Life Technologies) stained with 10µl of 500ng/ml ethidium bromide and visualised on a UVP ultraviolet transilluminator. Nucleic acid was quantitated using a Kontron Uvikon 860, or a Philips PU8620 spectrophotometer. An absorption of 1 at 260nm was taken to equal a concentration of 50mg/ml of double stranded DNA, 40mg/ml of single stranded DNA or RNA, and 33mg/ml of oligonucleotides.

2.4 POLYMERASE CHAIN REACTION (PCR)

2.4.1 PCR primers

PCR primers were designed to be complementary to the template sequence for at least 18 nucleotides, with equal proportions of A+T to C+G and if possible with a C or G at the 3' end. If required for cloning, additional non-complementary bases were added at the 5' end of the oligonucleotide to incorporate recognition sites for restriction enzymes in amplified products. Primers were synthesised by the Department of Molecular Pathology, Middlesex Hospital. The approximate annealing temperature of the primers was calculated according to the equation $T^0C = 2n(A+T) + 4n(C+G)$ where n equals the number of residues in the primer sequence. The sequences of primers used in this study are listed at the end of this section.

2.4.2 Reaction conditions

All PCR amplifications used approximately 50-500ng template DNA, 200µM dNTPs (Pharmacia), 1.5mM $MgCl_2$, buffer and enzyme according to the manufacturer's instructions (Promega) and 50pmol of each primer (forward and reverse) in a final volume of 20-50µl. The reaction was overlaid with mineral oil to prevent evaporation during the

temperature cycling. A control reaction containing all the components except template DNA was always included to rule out contamination. Reactions were carried out by denaturing initially for 3 minutes at 93^0C, followed by 30 seconds at the calculated annealing temperature, 30 seconds (for products under 750bp) or 1 minute (for larger products) at 72^0C and 30 seconds denaturing at 93^0C, for 30 cycles. A final annealing step was followed by 10 minutes at 72^0C to ensure completion of synthesis. Reactions were temperature cycled using a Hybaid Thermal Cycler. In the case of resistant or non-specific amplification, the conditions were altered by changing the annealing temperature, the amount of template, or the Mg^{2+} concentration. PCR products used for cloning were sequenced to check for fidelity of synthesis.

2.5 ENZYMATIC DNA MODIFICATION

2.5.1 Restriction digestion of PCR products

Unless otherwise specified, PCR products were first precipitated with salt and ethanol. After centrifugation at 13000 rpm in a microfuge, the pellet was washed in 70% ethanol and resuspended in the appropriate buffer. Restriction enzyme digestion was carried out according to the manufacturer's instructions. For termination and further purification, reaction products were either extracted with phenol or gel-purified.

2.5.2 Restriction digestion of genomic and plasmid DNA

Digestion of DNA was carried out in accordance with the enzyme manufacturer's instructions, in 0.5-2 x universal buffer (Stratagene) and an excess of enzyme (5-10 U/mg DNA). For digestion of genomic samples (except for reactions requiring very low salt conditions), spermidine was added to a final concentration of 4mM.

2.5.3 DNA ligation

Ligation of DNA fragments was carried out at 4^0C overnight, using T4 DNA ligase and buffer (containing ATP) at concentrations recommended by the manufacturer. For cloning into plasmids, where possible, non-complementary ends were used to enhance efficiency and determine orientation of the insert. Otherwise, to prevent intramolecular ligation, the vector molecule was pre-treated with calf intestinal alkaline phosphatase (Promega, 1unit

per 100pmol of ends) to remove 5'-phosphate groups (37^0C for 30-60 minutes under specified buffer conditions). Where necessary, phosphatase reactions were terminated by phenol extraction, or gel purification. For blunt end ligation, a vector to insert molar ratio of 3 was used, and for recessed ends, a ratio of 0.5-1. Reaction products were used directly for transformation of bacteria, and successful ligation determined by restriction digestion of purified plasmid DNA.

2.5.4 Blunting recessed ends

5' overhangs were flushed either with Klenow fragment of DNA-polymerase I or T4-polymerase in the presence of an excess of dNTPs (0.2mM), under recommended conditions. For 3' overhangs, in the presence of an excess of dNTPs, the 3' exonuclease activity of T4 polymerase was used to resect the overhang. Universal buffer (Stratagene) was used for all reactions at recommended concentrations.

2.6 NUCLEIC ACID ELECTROPHORESIS

2.6.1 DNA electrophoresis

DNA fragments were separated on 0.8% to 2% agarose (International Biotechnologies Inc.) gels with 500ng/ml ethidium bromide using 1 x TAE buffer. Fragment size was determined by comparison with HindIII digested λ DNA and H*ae*III digested ϕx174 DNA fragments (Promega). Agarose gels for Southern blot analysis or gels for preparation of DNA fragments, were electrophoresed at a voltage of <5V/cm for the necessary time to separate the DNA fragments under study. Samples were loaded into wells mixed with 6 x DNA loading buffer, and separated fragments visualised on a UV transilluminator.

2.6.2 RNA electrophoresis

RNA samples (up to 20μg in 5μl, denatured by heating to 65^0C, in 2μl 5 x RNA gel running buffer, 10μl deionised formamide, and 3.5μl concentrated formaldehyde, finally added to 2μl of RNA loading buffer) were separated on formaldehyde-agarose, consisting of formaldehyde (37% in water, 12.3M), and 1 x RNA running buffer added to melted agarose in water at 50-60^0C to a final percentage of 1-1.5%.

2.7 ISOLATION OF DNA FRAGMENTS FROM AGAROSE GELS

DNA fragments derived from PCR or restriction digestion reactions were separated by electrophoresis through a 1-2% agarose gel with 500ng/ml ethidium bromide in 1 x TAE. The required DNA fragment was visualised and excised from the gel under UV light, placed in an Eppendorf tube and snap frozen in liquid nitrogen. 1ml of silicon-based purification resin ('Magic/Wizard clean-up resin', Promega) containing guanidine hydrochloride was added to the thawed gel slice, and when dissolved was processed according to manufacturers instructions. DNA was quantitated by comparative ethidium bromide fluorescence.

2.8 SOUTHERN AND NORTHERN BLOT ANALYSIS

2.8.1 Blotting gels

Agarose gels for Southern blot analysis were incubated in 240mM HCl for 20 minutes to break large DNA fragments by acid depurination and then in denaturing solution (1.5M NaCl, 0.5M NaOH) for 60 minutes, changing the solution once. Formaldehyde gels for separation of RNA fragments were briefly washed in water prior to blotting. The gels were inverted and blotted onto Hybond N^+ (nylon) membrane (Amersham) in alkali transfer buffer (0.4M NaOH for DNA and 0.05M NaOH for RNA) overnight. Membranes were washed thoroughly in 2 x SSC before storage between acid-free tissue paper, or direct hybridisation.

2.8.2 Radiolabelling probes

Probes were radiolabelled by random priming of 30-50ng heat-denatured double stranded DNA (Random primed DNA labelling, Boehringer Mannheim Biochemica). Reaction conditions were according to manufacturers instructions. For each labelling reaction, 50mCi [^{32}P]-dCTP was added to the reaction mix, and incubated at room temperature for 1 hour.

2.8.3 Removal of unincorporated [^{32}P]-dCTP

A 1ml syringe was plugged with polymer wool, filled with Sephadex G50 (DNA grade), and packed by centrifugation for 2 minutes at 300*g*. The probe labelling reaction was made up to a final volume of 100µl with 2 x SSC, and centrifuged as before. The activity of the recovered probe was measured by counting 2ml using a Bioscan QC 2000 β-counter.

2.8.4 Prehybridisation and hybridisation of membranes

Membranes were wetted in 2 x SSC before being rolled into glass hybridisation bottles (Hybaid) with mesh (Hybaid) interleaved. 10ml of hybridisation solution were added and the bottles rotated in a Hybaid oven at 42^{0}C for at least 3 hours. Probes were denatured at 98°C for 5 minutes and added to the hybridisation solution at 10^6 dpm/ml. Hybridisations were incubated at 42^{0}C for 4 to 16 hours, depending on the blot.

2.8.5 Washing membranes after hybridisation

Membranes were washed three times in 2 x SSC/0.1% SDS at room temperature for 20 minutes and then in solutions with 2xSSC, 1xSSC or 0.5xSSC all with 0.1% SDS, dependent on the probe used at 65^{0}C for 30 minutes.

2.8.6 Autoradiography

Membranes were exposed to X-ray film (XAR-5, Kodak) at -70°C with two intensifying screens (Lightening Plus, Cronex, Dupont) for between 1 hour and 14 days. Films were developed on a Fugi RGII film processor.

2.8.7 Stripping membranes

Membranes were stripped of annealed probe by incubating in 2mM EDTA/1mM Tris-HCl (pH8.0)/0.1% SDS at 98^{0}C until the solution had cooled to room temperature. The stripping procedure was checked by exposure to X-ray film as before.

2.9 DNA SEQUENCING

2.9.1 Sequencing using the 'Sequenase' kit version 2.0 (USB)

Template DNA for sequencing was double stranded plasmid, irreversibly denatured by addition of 3.5 parts 1M NaOH / 1mM EDTA pH7.9, to 15 volumes of DNA (1mg/ml) in solution. Denatured template for sequencing was equilibrated with TE in a spin column, and stored at -20^0C. Sequencing was carried out using the 'Sequenase' kit version 2.0 as detailed in USB protocols.

2.9.2 Sequencing gels

Sequencing reactions were analysed on 6% polyacrylamide (Accugel 40, National Diagnostics) 8M urea denaturing gels in 1 x TBE, using wedged spacers and BRL sequencing equipment according to the manufacturers instructions. Gels were dried on a vacuum drier and exposed to autoradiographic film (Kodak XAR-5) for 1-14 days at room temperature.

2.9.3 Preparation of nuclei and DNAase1 sensitivity analysis

Nuclei were isolated by suspension of washed cells in hypotonic buffer (RSB) and NP-40 (0.3% (v/v)), at 4^0C. Nuclei were washed twice in cold RSB, resuspended at 10^8/ml in RSB, and 2.10^7 incubated for 5 minutes at 37^0C with DNAase1 at increasing concentration (0-20μg/ml). Reaction was terminated by addition of 400μl of 1 x DNA lysis buffer.

2.10 PREPARATION AND ANALYSIS OF PROTEIN

2.10.1 Preparation of proteins for Western blotting

For preparation of crude protein extracts, washed cells were pelleted and resuspended at 2.10^8 cells per ml in break buffer consisting of 6mM Pipes pH7.3, 6% (w/w) sucrose, 60mM KCl, 1.8mM NaCl, 2.3mM $MgCl_2$, 1μM di-isopropylfluorophosphate, 1mM PMSF, and 1μg/ml TLCK. Cells were disrupted by 2 x 5 second bursts of sonication (MSE 150W sonicator), mixed with an equal volume of 2 x SDS reducing buffer, and boiled for 10 minutes. Insoluble cell debris was pelleted in a microfuge. Alternatively,

crude extracts were made by detergent lysis. For this, 100µl of cell lysis buffer (1% Triton x-100 (v/v), 10mM HEPES, 3.5mM $MgCl_2$, 1mM PMSF, 0.1mM leupeptin) was added to 10^7 cells and incubated on ice for 30 minutes. Insoluble debris including intact nuclei were pelleted in a microfuge, and 2 x SDS loading buffer added to the supernatant. Protein concentrations were determined by a dye-binding assay (Bio-Rad) as described (Bradford, 1976).

2.10.2 Western blot analysis

Proteins were separated on 7.5-12% SDS polyacrylamide denaturing gels at 40V and room temperature using Bio-Rad II gel apparatus according to the manufacturer's instructions. The electrophoresed proteins were transferred to Hybond C nylon membranes (Amersham International) using a semi-dry blotter (Bio-Rad) according to the manufacturer's instructions. The membrane was blocked with superblock (5% non-milk fat (Marvel), 1% BSA, 5% FCS, 1M glycine, 0.01% Tween-20, in PBS) for 2-4 hours at room temperature, or 4^0C overnight. Membranes were washed briefly in PBS-T before being incubated with an appropriate dilution of antiserum in TTF at room temperature for 1-3 hours or overnight at 4^0C. After washing 4 times over 30 minutes in PBS-T, the membrane was incubated with horse-radish peroxidase-conjugated second antiserum in TTF for 1-2 hours. Membranes were again washed 4 times in PBS-T, and proteins detected using an ECL system (Amersham International) with film exposure times ranging from 30 seconds to two hours.

2.11 TISSUE CULTURE

2.11.1 Culture of cell lines

Unless otherwise indicated, all suspension cell lines were grown in RPMI 1640 (Life Technologies) supplemented with 10% (v/v) FCS (Globepharm), 2mM L-glutamine (Life Technologies), 100iu/ml penicillin, and 100mg/ml streptomycin (Life Technologies) at 37^oC under 5% (v/v) CO_2. Adherent cell lines were grown in DMEM (Life Technologies) supplemented as above at 37^oC under 10% (v/v) CO_2. Trypsin-EDTA (Gibco) was used to loosen adherent cells.

2.11.2 Induction of myeloid cell lines

Myeloid cell lines were induced to differentiate with 10^{-6}M all-*trans* retinoic acid, 5.10^{-8}M 1,25$(OH)_2$-vitamin D3 (Calbiochem), 1.25% DMSO, 5.10^{-8}M PMA, or 500u/ml IFN-γ. Differentiation was recognised by changes of cell morphology and adherence, decreased cell growth (except with IFN-γ), and increase in activity of the NADPH-oxidase, indicated by positive NBT staining.

2.11.3 Purification and culture of primary human cells

Leukocyte rich serum was obtained by sedimentation of red cells from 5-50mls of heparinised blood in the presence of 1% final volume of Dextran (10% (v/v) in 150mM NaCl). This was overlayed on one third volume of Ficoll/Hypaque (density 1.077g/ml), and was centrifuged at room temperature for 20 minutes at 1000*g*. Interface cells were recovered, washed with 10-40ml PBS, and centrifuged three times at 200*g* to separate platelets. Finally, cells were adhered to tissue culture plastic for 1-2 hours, and non-adherent cells (mainly lymphocytes) washed away. Cultures were maintained in RPMI1640 supplemented with heat-inactivated 2-5% FCS, 2mM glutamine, and antibiotics. Neutrophils were recovered from the Ficoll pellet after hypotonic lysis of residual contaminating red cells (pellet agitated in 10-25ml ddH_20 for 10 seconds, followed by equal volume of 0.3M NaCl to restore tonicity) and were used immediately for preparation of protein extracts, or measurement of NADPH-oxidase activity.

Adult human bone marrow was treated in a similar way. Whole marrow was diluted 1:1 with unsupplemented RPMI. This was fractionated over Ficoll, and interface cells treated as above. Non adherent cells (containing mainly lymphocytes and bone marrow progenitor cells) were washed away from monocytes, and used for transduction experiments.

2.11.4 Long term storage of cells

Cells were frozen at 2.10^7/ml in growth media with 20% (v/v) FCS and 10% (v/v) DMSO by first cooling slowly to -70^{0}C in a polystyrene box overnight and transferring to liquid nitrogen for long term storage. To thaw, cells were warmed quickly at 37^{0}C, washed and pelleted by centrifugation and resuspended in the appropriate medium.

2.12 CELL TRANSFECTION

2.12.1 Calcium phosphate precipitation

6-16 hours before transfection, adherent cells were trypsinised and replated at a density of 5.10^5-5.10^6 per 9cm tissue culture dish (depending on cell type). For transfection, 10-30μg of plasmid DNA was mixed with sterile ddH2O to a volume of 450μl and mixed with 50μl of sterile 2.5M $CaCl_2$. This was added dropwise to 0.5mls of sterile 2 x HBSS (280mM NaCl, 1.5mM Na_2HPO_4, 50mM HEPES (sodium salt), pH7.05+/–0.05) through which a constant stream of air was bubbled. The mixture was allowed to stand for 20-30 minutes, applied dropwise to the cell culture medium, and incubated at 37^0C for 4 hours to overnight. Formation of a fine precipitate was noted by light microscopy. Cells were then washed three times with PBS and fed with fresh medium. Sometimes, for resistant transfections, at the end of incubation with cells, the transfection mixture was removed and replaced with 1 x HBSS containing 15% (v/v) glycerol for 2 minutes, before being thoroughly washed and refed with complete medium. For modified calcium phosphate transfection (Chen et al. 1987), 10-30μg of plasmid DNA was mixed with 0.5mls of 0.25M $CaCl_2$, and added to 0.5mls of 2 x BBS (2 x BES-buffered saline, containing 50mM BES pH 6.95, 280mM NaCl, 1.5mM Na_2HPO_4). This was incubated at room temperature for 20 minutes, added dropwise directly to the cell culture medium (DMEM supplemented with 10% FCS (v/v) and antibiotics), and incubated at 37^0C at 2-4% CO_2 overnight. The next day, cells were refed with fresh medium and incubated under normal conditions.

2.12.2 Liposome-mediated transfection

Liposomal transfections were carried out according to manufacturers instructions (Lipofectin, Gibco). Briefly, equal volumes of serum and antibiotic-free medium (Optimem, Gibco) were mixed with DNA or liposomes at an optimised ratio of between 1:2 and 1:10 respectively. The combined mixture was incubated for 20 minutes at room temperature, and added directly cells (adherent or non-adherent) for a period of 4 hours to overnight. Cells were refed with complete medium after transfection.

* Unless otherwise stated, transfection efficiency was determined by co-transfection of pSVβGal, and extracts normalised for this activity prior to CATassay. All measurements of CAT synthesis were within the limits of a linear calibration curve, and are represented as units of optical density (405nm), adjusted for background activity obtained from untransfected cell extracts.

2.12.3 Electroporation

10^7 cells were washed three times with PBS at room temperature, resuspended in 0.25ml of retained medium, and transferred to a 0.4cm sterile electroporation cuvette. 20-50μg of DNA was mixed with the cells, which were allowed to incubate at room temperature for 20 minutes. Cells were gently resuspended prior to electroporation (0.25kV, 960μF, Bio-Rad Gene Pulser), and after a further 20 minutes resting at room temperature, were gently transferred into 5 ml of conditioned medium using a sterile wide bore pipette, and incubated overnight at 37^0C. The next day cells were refed with fresh medium and in some cases equally split into separate flasks for stimulation experiments. Reporter gene activity was measured 48-72 hours after electroporation.

2.12.4 Detection of reporter gene expression

For detection of reporter gene expression, cells extracts were prepared by repeated freeze-thaw, or detergent lysis according to manufacturers instructions. CAT protein was quantitated by colourimetric immunoassay (CAT ELISA, Boehringer Mannheim Biochemica), and directly stained in cells by indirect immunofluorescence (5Prime-3). β-galactosidase was detected in cell extracts by colourimetric assay for enzyme activity (Promega). For cell staining, cells were fixed for 15 minutes in 0.5% glutaraldehyde, and washed twice with PBS. Sufficient X-Gal solution (2mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% (v/v) NP40, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, and 0.1% X-gal added just before use) as added to cover cells, which were incubated for 1-4 hours at 37^0C. Cells were washed, and staining visualised by light microscopy.

2.13 PRODUCTION AND TITRATION OF VIRUSES

2.13.1 Production and titration of retroviruses

Retrovirus producer cell lines were created by calcium phosphate transfection of subconfluent (30-50%) packaging cell lines (PA317, AM12, ΩE) with 10μg of vector plasmid per 9cm plate. If the vector did not contain a selectable marker gene, 0.5-1μg of a separate plasmid encoding resistance to G418 (pSV2Neo) was co-transfected with the vector. After 48 hours, cells were harvested and replated at 1-20% density in fresh

medium to which G418 was added (1mg/ml). After 14-21 days, colonies were isolated by ring cloning, and passaged from 6 well plates, eventually to 9cm dishes. Cells were then grown in the absence of drug selection. When confluent, cells from each clone were refed with fresh medium for 12-24 hours, after which time the medium was harvested and frozen at -70^0C, and cells frozen in liquid N_2. For a polyclonal producer cell line, the cloning steps were omitted. To titre virus stocks by expression of drug resistance, frozen supernatants (1-100μl) were thawed and used in dilution to infect 10^5 3T3 cells freshly plated in 6 well plates, in the presence of polybrene (8μg/ml). After 48 hours, cells were split into fresh medium containing G418 (1mg/ml), at 1-50% of final density. Titre was estimated by growth of resistant colonies. For titration of viruses not incorporating a selectable marker, 10^6 3T3 cells freshly plated on a 9cm dish were infected with dilutions of virus supernatant (0.01-10ml) and grown to confluence. If possible, a vector of known titre encoding the same gene in addition to a selectable marker was used in a parallel 9cm dish. At confluence, genomic DNA was prepared, digested with appropriate restriction enzymes, and blotted to nylon. Comparison of signal obtained by probing for the shared gene provided an estimate of titre. Alternatively, known molar dilutions of vector plasmid, digested with the same enzyme, were run on the same gel. High titre clones were thawed and expanded in selection as before.

2.13.2 Production and titration of adeno-associated viruses

For conventional production of recombinant adeno-associated virus (rAAV), 5.10^6 human embryonal kidney 293 cells were plated in 9cm dishes 16-20 hours before calcium phosphate transfection with vector and packaging plasmid (usually 10μg of each). Cells were then gently washed with PBS and fresh medium added containing wild-type human adenovirus 5 at a multiplicity of infection of ~5. Cells and supernatant were recovered after 48-72 hours at completion of the cytopathic effect, at which time most cells were detached. rAAV was recovered by 4 freeze-thaw cycles, followed by low speed centrifugation and filtration through a 1.2μ membrane to remove debris. For heat inactivation of adenovirus, supernatants were placed in a water bath at 56^0C for 10 minutes to 1 hour. rAAV supernatants were stored at -70^0C.

2.13.3 Production and titration of adenoviruses

Recombinant virus was rescued 'in *vivo*' by calcium phosphate co-transfection of a full length human adenovirus 5 genome and a vector genome (20μg of each) into freshly plated 293 cells. After 4-6 hours, the medium was removed and cells overlaid with MEMF11 (MEM + 10% FCS) in 1% agarose. When the agarose had solidified, plates were incubated for 7-10 days at 37^0C until plaques appeared. Individual plaques were picked by punching out agar plugs with a sterile Pasteur pipette, and were stored in 0.5ml PBS with 10% (v/v) glycerol at -70^0C. Recombinant virus was amplified on 293 cells, and checked for vector sequences by restriction digestion of purified viral DNA with appropriate enzymes. To make high titre stocks, at the end of the cytopathic effect, cells and medium were centrifuged at 800*g* for 15 minutes, and the pellet resuspended in 2ml PBS with 10% glycerol per 9cm dish. This was subjected to 4 freeze-thaw cycles, and stored at -70^0C. For a crude estimate of viral titre, 3.10^4 293 cells were added to dilutions of virus in 100μl of complete medium, and plated in a 96 well dish in triplicate. Titre was estimated from the first dilution at which the cytopathic effect was partial at 2-3 days (cytopathic end-point assay). A more accurate assessment of titre was obtained from conventional plaque assay. For this, confluent 293 cells were infected with dilutions of virus in 6cm dishes, and overlaid with MEMF11 as above. Plaques were counted after 4-7 days. Amplification and titration of wild-type adenovirus was carried out by the same methods, except that HeLa cells were sometimes used for titrations.

2.13.4 Density gradient centrifugation

Crude virus supernatants (~5ml) were layered over a CsCl step gradient (3ml of 1.25g/ml, and 2ml of 1.45g/ml in TD), and centrifuged at 35,000rpm in a Beckman SW41 Ti swing out rotor for 80 minutes at room temperature (Beckman L8-M ultracentrifuge). The interface was recovered by side or bottom puncture, and mixed with CsCl in TD to fill the tube to a final density of 1.36g/ml. This was centrifuged overnight at 4^0C and 40,000 rpm in the same rotor and the lower band (containing AAV) recovered by side puncture. The upper band (containing adenovirus) was discarded. Finally, the virus containing fraction was dialysed three times against PBS at 4^0C.

2.14 DETECTION OF NADPH-OXIDASE ACTIVITY

2.14.1 Detection by NBT cell-staining

Non-adherent neutrophils, transformed myeloid cells, or adherent monocytes were washed three times with PBS, and incubated for 30 mins in NBT reagent (nitroblue tetrazolium (NBT) 1mg/ml, PMA 0.1-1μg/ml in PBS). Positive cells were scored by light microscopy, by their ability to reduce NBT to dark blue staining formazan.

2.14.2 Detection by ferricytochrome c reduction

Quantitative assay for production of superoxide was carried out by reduction of ferricytochrome c. For this assay, 5.10^6 washed cells were resuspended in HBSS with calcium and magnesium at a final concentration of 2.10^6 cells per ml, in the presence of 100μM ferricytochrome c and 150μM NADPH. Reaction was initiated by the addition of PMA (1μg/ml), and incubated at 37^0C with gentle mixing. 0.5ml aliquots were removed from the reaction at 10 minute intervals, and quenched by addition of 1ml of ice-cold PBS, and centrifugation at 4000rpm in a microfuge at 4^0C. A parallel reaction was incubated in the presence of SOD (50μg/ml), and SOD-inhibitable reduction of cytochrome c measured by differential absorbance at 550nm and 557nm (isobestic points) in a dual beam spectrophotometer (Uvikon 860, Kontron). Superoxide production was calculated based on an extinction co-efficient for cytochrome c at 550nm of $21.1 mM^{-1}.cm^{-1}$.

2.14.3 Detection by chemiluminescence

For chemiluminescence-based assay of NADPH-oxidase activity, 5.10^6 cells were washed twice with PBS, and resuspended in a total volume of 1ml HBSS with calcium and magnesium (0.5 mM and 1mM, respectively), 10μM luminol and 10U/ml horse radish peroxidase. Activation was initiated by the addition of PMA (1μg/ml) at 37^0C, and superoxide production measured on either a Berthold 953 luminometer (chapter 4) or a LKB452 luminometer (chapters 5 and 6).

2.15 CELL-FREE ASSAY

2.15.1 Preparation of cytosolic and membrane fractions

Cells were resuspended at a concentration of 2.10^8 cells per ml in break buffer consisting of 6mM Pipes pH7.3, 6% (w/w) sucrose, 60mM KCl, 1.8mM NaCl, 2.3mM $MgCl_2$, 1μM di-isopropylfluorophosphate, 1mM PMSF, and 1μg/ml TLCK. Cells were sonicated on ice for 2 bursts of 5 seconds using a MSE 150W sonicator. Sonicate was centrifuged at 250,000*g* at 2^0C for 45 minutes on a discontinuous sucrose gradient of 0.5ml 15% on 0.5ml 34% (w/w), in a Kontron TST55.5 rotor. Cytosol was collected above the 15% sucrose, and membranes at the interface. Collected membranes were diluted 1:2 in ice-cold break buffer, pelleted by centrifugation as above, and resuspended at a concentration of 2.10^8 cell equivalents per ml of solubilization buffer (120mM sodium phosphate pH7.4, 1mM $MgCl_2$, 1mM EGTA, 1mM dithiothreitol (DTT), 20% glycerol, and 40mM octyl glucoside. This was homogenised on ice, and centrifuged at 48,000*g* for 30 minutes to remove insoluble debris. Supernatant was dialysed overnight at 4^0C against solubilisation buffer lacking octyl glucoside, and was frozen at -70^0C or used directly for cell-free activation.

2.15.2 Cell-free NADPH-oxidase activation and detection

Solubilised membrane protein was mixed with cytosol in the presence of varying concentrations of SDS (40-120μM), and incubated at room temperature for 2 minutes before addition of assay buffer (65mM sodium phosphate buffer, pH7.0, 1mM EGTA, 1mM $MgCl_2$, 10μM FAD, 10U/ml horse radish peroxidase, and either 10μM luminol or 100μM cytochrome c depending on the detection method), and finally NADPH (0.2mM) to initiate the reaction. Reactions were incubated at 37^0C, all solutions pre-warmed, and measured on a Berthold 953 luminometer as before or in a double-beam spectrophotometer (Uvikon 860, Kontron). For the latter detection system, the reference sample was identical, but was supplemented with superoxide dismutase (SOD 50μg/ml). SOD-inhibitable cytochrome c reduction was estimated by differential absorbance at 550nm and 557nm.

2.16 SOLUTIONS

Double distilled water (ddH_2O) was used to prepare all solutions. Solutions were autoclaved at 121^{0}C for 20 minutes unless otherwise stated.

Ampicillin stock 50mg/ml in water, filter sterilised, working concentration 50μg/ml.

Assay buffer (for cell-free assay)

65mM sodium phosphate pH7.0, 1mM EGTA, 1mM $MgCl_2$, 10μM FAD, 10U/ml horse radish peroxidase.

BBS (2x) 50mM BES pH 6.95, 280mM NaCl, 1.5mM Na_2HPO_4.

Break buffer 6mM Pipes pH7.3, 6% (w/w) sucrose, 60mM KCl, 1.8mM NaCl, 2.3mM $MgCl_2$, 1μM di-isopropylfluorophosphate, 1mM PMSF, and 1μg/ml TLCK.

Calcium chloride 36.8g $CaCl_2.2H_2O$ made up to 100ml with ddH_2O.

Cell lysis buffer (for proteins)

1% (v/v) Triton X-100, 10mM HEPES, 3.5mM $MgCl_2$, 1mM PMSF, 0.1mM leupeptin.

Denaturing solution 1.5M NaCl, 0.5M NaOH.

Denhardts solution (100x)

20g Ficoll 400 (Pharmacia), 20g polyvinylpyrrol-idone, 20g BSA (Fraction V), water to 1 litre. Sterilised by filtering and stored at –20°C.

DEPC-treated water

DEPC added to 0.1% (v/v), incubated overnight at room temperature and autoclaved.

DNA loading buffer (6x)

1.5g Ficoll in 10ml water, bromophenol blue, xylene cyanol.

DNA lysis buffer 10mM Tris-HCl pH8.0, 5mM EDTA, 0.5% (w/v) SDS, 150µg/ml proteinase K added just before use.

HBSS (2x) 280mM NaCl, 1.5mM Na_2HPO_4, 50mM HEPES (sodium salt), pH7.05+/–0.05.

Hirt buffer (10x) 100mM Tris-HCl pH7.5, 6% SDS (w/v), 100mM EDTA, proteinase K 100mg/ml, yeast tRNA 50-100mg/ml.

Hybridisation solution

('Starks') 1 x Denhardts, 150mg/ml sonicated salmon sperm DNA (denatured at 98°C for 5 minutes), 5 x SSC, 0.5% SDS, 50% deionised formamide, 20mM phosphate buffer.

LB (Luria-Bertani) bacterial growth medium

10g Bactotryptone (Difco), 5g bacto yeast extract (Difco), 10g NaCl, water to 1 litre. 15g/litre bacto-agar (Difco) added for LB agar plates.

RSB 10mM Tris-HCl pH7.4, 3mM $MgCl_2$, 10mM KCl.

SDS protein sample reducing loading buffer (2x)

The following were added to 40ml of ddH_2O: 1.52g Tris base, 20ml glycerol, 2g SDS, 2ml 2-mercaptoethanol, 1mg bromophenol blue (BDH). 1M HCl was added until the pH was 6.8 and the solution made up to 100ml with ddH_2O.

Sephadex G50 10g Sephadex G50 (Pharmacia) was incubated in 100ml 2 x SSC at 65°C for 3 hours, replacing the supernatant with new SSC during the incubation. Stored at 4°C.

SSC (20x) 3M NaCl, 0.3M sodium citrate.

Superblock 5% non-milk fat (Marvel), 1% BSA, 5% FCS, 1M glycine, 0.01% Tween-20, in PBS.

Terrific broth 12g bactotryptone, 24g bacto-yeast extract, 4mls glycerol added to 900 mls H_2O, sterilised by autoclaving. When cool, 100 mls of autoclaved 0.17M KH_2PO_4, 0.72M K_2HPO_4 was added.

TAE (50x) 0.2M Tris base, 1M glacial acetic acid (BDH), 50mM EDTA, pH8.0.

TBE (10x) 0.9M Tris base, 0.9M Boric acid, 1mM EDTA, pH8.0.

TD buffer 8g NaCl, 0.38g KCl, 0.1g Na_2HPO_4, Tris HCl, pH 7.8.

TE (pH8.0) 10mM Tris-HCl, pH8.0, 1mM EDTA.

TTF 200mM Tris-HCl, pH7.5, 0.5% Tween, 10% FCS (Globepharm).

Transfer buffer 48mM Tris-HCl, pH9.2, 39mM glycine, 20% methanol (BDH).

X-Gal 20mg/ml in dimethylformamide, stored at -20°C.

X-Gal solution 2mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP40, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, and 0.1% X-Gal added just before use.

2.17 *PCR* PRIMERS

Sequence of primers used for PCR

Name	Sequence	Comments
p47(6)	GCGATGCATCAGTGATAATG	NsiI site
p47(9)	CGCGTCTAGACTGGGTGGCCTCCAG	XbaI site
p47(11)	CAAAAGCTTTGAGGCAGGAGCAGC	HindIII site
p47(12)	TGCAAGCTTTTACACCCCTGCAAGCC	HindIII site
p47(26)	GTCAAGCTTGCGACAAAAGCGACT	HindIII site
p47(27)	TTATAAAGCTTGGAAGGCTTCTCGGAAG	HindIII site
p47(28)	TGAATCTAGAGCTGCTCCTGCCTCAGGGCC	XbaI site
PM1	GAGCACTGGAGGCCACCCAGTC	
PM5	GTTTTATGGAACTCGTAGATCTCG	

CHAPTER 3:

THE PROMOTER REGION OF THE p47^{phox} GENE

3.1 INTRODUCTION

For gene transfer to be therapeutically successful, expression must be sufficient to sustain the desired clinical effect. This may be achievable using heterologous promoter systems, but fully regulated physiological gene expression is preferable, and particularly important for genes which encode active participants in control of cell cycle or cell differentiation. The following preliminary studies were designed to investigate some of the mechanisms that direct both myeloid and differentiation-specific expression of the p47^{phox} gene.

3.1.2 Defining functional transcription units

Basal promoter elements for most protein-coding genes in mammalian cells lie within the first 200bp upstream of the transcription start site. Although necessary for transcription, and often able to direct some tissue-specific or developmental specificity, these regions in isolation are rarely sufficient to support fully regulated high level expression. For this to occur, the basal promoter interacts with distally occurring elements. Enhancer elements are characterised by the ability to potentiate transcription independently of orientation, and distance from initiation site. However, isolated enhancer elements invariably show diminished activity when introduced at ectopic integration sites in comparison to the natural site, and many integration events will fail to support desired levels of gene expression. This may in part reflect the need for multiple enhancer elements to function in a co-operative rather than hierarchical manner, but extensive evidence now indicates that different regions of chromatin have different properties, and that these properties as well as individual regulatory elements are critical determinants of local gene expression.

Functional expression domains in chromatin have been defined by characteristic properties that distinguish them from bulk chromatin, including sensitivity to DNAase1

(Weisbrod, 1982; Reeves, 1984). In contrast to bulk chromatin which is relatively resistant to DNAase1, genes become preferentially susceptible in tissues in which expression occurs. This does not reflect transcription itself, but the potential for active transcription, and always extends over the whole transcribed region and often some distance beyond. The basis for nuclease sensitivity is not clear, but is associated with alterations of nucleosomal structure including depletion of histone H1, and histone acetylation (Lewin, 1994). Superimposed on regional sensitivity, are hypersensitive sites which are likewise are usually related to potential for gene expression in specific cells or tissues, rather than active transcription (Gross and Garrard, 1988). In some circumstances, however, events critical for induction of transcription result in the appearance of new hypersensitive sites (Beato, 1989). They may lie long distances upstream of the start of transcription, but may also be found within intronic or 3' untranslated regions of the gene, and represent segments of so-called 'open chromatin' within which the structure and/or the dynamics of nucleosome assembly are altered.

The state of gene methylation also correlates with potential for gene transcription, rather than the active process. Most of the methyl groups added to eukaryotic DNA occur at the 5 position of cytosine residues in the dinucleotide CG (5-meC), the majority of which are methylated (Razin and Riggs, 1980; Bestor and Coxon, 1993). Genomic under-representation of CG (frequency 0.008) compared to GC (frequency 0.04) and methylation are probably linked. In contrast to oxidative deamination at the N4 position of 5-meC which forms a T residue, deamination of un-methylated C forms uracil which is readily recognised, and excised by DNA repair mechanisms. Retention of methylated CG may therefore be a reflection of associated gene function. Most CG sites are always either methylated or un-methylated, but a few exhibit tissue-specific variability in which undermethylation is a feature of domains which contain transcribed genes, and which are significantly DNAase1 sensitive (Cedar, 1988).

In some regions of DNA known as CpG-rich islands, the density of CG dinucleotides approaches the predicted value (Bird, 1986). These have an average G-C base pair content of 60% compared with the 40% average of bulk DNA, and are stably unmethylated. Although many of the genes associated with CpG islands are

constitutively expressed 'housekeeping' genes, some tissue-specific genes are linked to islands which are unmethylated irrespective of the state of expression of the gene. The functional significance of methylated DNA is controversial, particularly as yeasts and invertebrates such as *Drosophila* and the nematode *C.elegans* lack meC (Uriel-Shoval et al. 1982), but it has been implicated in the control of a number of cellular processes in eukaryotes, including transcription (Busslinger et al. 1983; Boyes and Bird, 1991), genomic imprinting (Swain et al. 1987), developmental regulation (Antequera et al. 1989), mutagenesis (Cooper and Youssoufian, 1988), DNA repair (Hare and Taylor, 1985), X-inactivation (Pfeifer et al. 1990), and chromatin organisation (Lewis and Bird, 1991). Abnormal methylation of an expanded triplet repeat through which FMR-1 gene transcription is silenced in Fragile X syndrome (Sutcliffe et al. 1992), and aberrant promoter methylation of tumour suppresser genes associated with tumorigenesis (Sakai et al. 1991) are both significant indicators of a prominent role in gene activation and mutation. Furthermore, the DNA methyl transferase gene has been shown to be essential for the normal embryonic development of mice (Li et al. 1992), and to be associated with DNA replication foci (Leonhardt et al. 1992).

Locus control regions (LCR) are either single or clusters of sites which are necessary for proper expression of a linked gene or genes. They are defined by gene expression in a transgenic system that is insensitive to position of integration, and dependent on the number of copies of the transgene. The human β-globin LCR is the paradigm, and is localised to 4 DNAase1 hypersensitive sites, corresponding to transcription factor binding-sites, within a region of about 15kb (Grosveld et al. 1987; Dillon and Grosveld, 1993). Full LCR activity can be achieved by joining shorter fragments, but full level and regulated expression is dependent on linkage to the globin promoter. LCR function is tissue specific, and in non-erythroid tissues is susceptible to position effect. Domains may also be defined both physically and functionally by boundary elements, including matrix attachment sites (MARs), at which points DNA is attached to the nuclear matrix, and insulator sequences which prevent gene regulatory influences from crossing domain boundaries (Kellum and Schedl, 1991; Chung et al. 1993; Lewin, 1994). The boundary regions of the chicken lysozyme gene, which is located within a 21kb chromatin domain of elevated DNase1 sensitivity flanked by two MARs, have been functionally

characterised (Bonifer et al. 1994). The entire locus is expressed at high level and independent of chromosomal position in macrophages of transgenic mice. Position independent expression is, however, lost if one of the tissue-specific enhancer regions is deleted, although tissue-specificity is largely retained. Deletion of boundary regions has no effect on copy number-dependency, but increases the incidence of ectopic expression, and are therefore necessary to suppress transgene expression in inappropriate tissues. Whether LCR regions and boundary elements are necessary for the regulation of all genes is unknown.

3.1.3 Regulation of NADPH-oxidase gene expression

Gene regulation for all components of the NADPH-oxidase is poorly understood. The most extensively investigated component is gp91^{phox}. The nucleotide sequence of the proximal promoter region, and partial characterisation of *cis*-elements and *trans*-acting factors involved in regulating tissue-specific expression have been described (Skalnik et al. 1991a). One feature of particular interest is a duplicated CCAAT box between -106bp and -124bp, of which the distal motif is recognised by the classical, and ubiquitous CCAAT-binding factor (CP1). A second protein, CCAAT displacement protein (CDP), was also found to bind to the region surrounding this motif, but in contrast was expressed primarily in cells in which the gp91^{phox} gene is transcriptionally inactive. CDP-binding activity is therefore associated with repression of gp91^{phox} transcription, and must be disrupted for induction of myelomonocytic expression of the gp91^{phox} gene. Functionally, a 450bp fragment of the proximal promoter was sufficient to target reporter transgene expression to murine monocytes and macrophages, but not to other phagocytic cells that express endogenous gp91^{phox}, implying that other *cis*-acting elements are necessary for fully regulated expression (Skalnik et al. 1991b). Other regions of interest within 1.5kb of the initiation site include an Alu repeat between -930 and -650, and several regions of simple sequence. No significant homology was identified with 5' flanking regions of other characterised myeloid specific genes, including cathepsin G, myeloperoxidase, and neutrophil elastase.

Regulation of gene expression by cytokines has only been studied in detail for IFN-γ. Specifically, IFN-γ has been shown to induce levels of gp91^{phox} mRNA *in vitro* in

normal neutrophils, monocyte-derived macrophages and the promonocytic cell-line THP-1 (Newberger et al. 1988; Cassatella et al. 1989,1990). In the latter two cell types, increased abundance of gp91^{phox} mRNA occurred largely as a result of an increase in the rate of transcription, although levels of immunoreactive protein were little changed. Signal transduction pathways that mediate the action of IFN-γ on gp91^{phox} gene expression are not clear, but seem to be specific, and independent of those that mediate changes in expression of other oxidase components (Amezega et al. 1992). Regulation of gene expression for p22^{phox} does not seem to be influenced by IFN-γ.

3.2 CHARACTERISATION OF THE P47^{phox} PROMOTER

Expression of the cytosolic component p47^{phox} is regulated in a differentiation and tissue-specific manner, and is found almost exclusively in terminally differentiated phagocytic cells. This is reflected in the marked changes of expression that accompany differentiation of immature myeloid cell lines, such as HL60, to a mature phenotype (Rodaway et al, 1990). As discussed previously, moderate levels of p47^{phox} protein are expressed in EBV-immortalised B cells (levels of messenger RNA are equivalent to that of induced HL60 cells), and low levels in uninduced HL60 cells, and the Daudi B cell-line. Although tissue distribution and induction of expression during cell differentiation closely mirror gp91^{phox}, regulatory mechanisms seem to be independent (Cassatella et al, 1991; Amezega et al. 1992). Regulation of expression by cytokines is less clearly established than for gp91^{phox}, although IFN-γ has been reported to down-regulate p47^{phox} gene transcription in mature neutrophils (Cassatella et al. 1991). More convincingly, retinoic acid has been shown to induce transcription of p47^{phox} in HL60 cells *in vitro*, determined by cycloheximide insensitive run-off transcription assay, and may have a direct regulatory function *in vivo* (Rodaway et al. 1990).

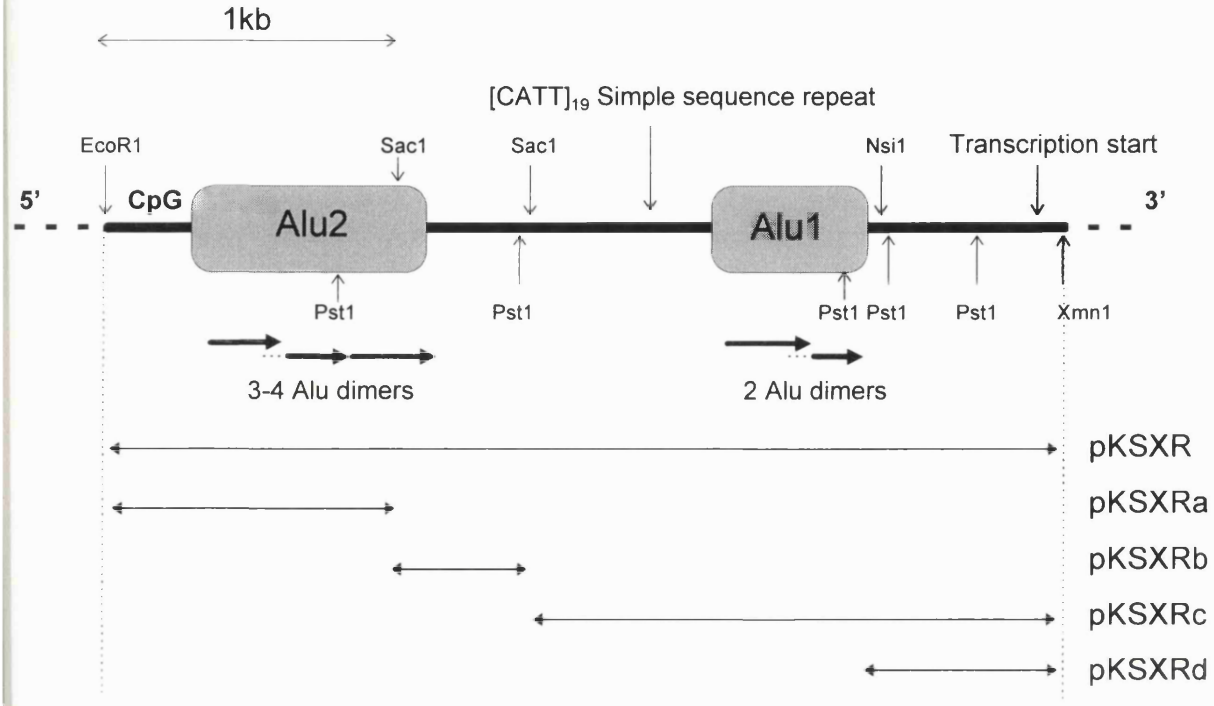


Fig 3.1. Schematic of p47^{phox} promoter. A 3.2kb EcoR1 (–3100bp) to Xmn1 (+71bp) fragment of genomic DNA incorporating the proximal promoter of the p47^{phox} gene, and cloned into the EcoR1 and Sma1 sites of pBluescript II KS (pKSXR), was mapped by restriction digestion and direct sequencing. Subfragments and vectors were generated by digestion with Sac1 (Sac1 site in polylinker of pKS 3' to Xmn1/Sma1 fusion), religation (pKSXRa), and cloning of the two smaller digestion products into pKS (pKSXRb and pKSXRc), and digestion with EcoR1 and Nsi1 followed by religation of blunted ends (pKSXRc). Alu sequences are shown by the shaded boxes, with an arrow to indicate orientation and position of possible dimers. The distal 350bp is over-represented for the dinucleotide CpG, which is indicated. A simple sequence repeat occurs between the two blocks of Alu sequence.

3.2.1 Sequence analysis of the p47^{phox} promoter.

DNA sequence analysis was performed on a previously uncharacterised 3.2kb EcoR1(-3100bp) to Xmn1(+71bp) genomic fragment (XR) isolated from a library cosmid pCos2.1, and cloned into a Bluescript vector KS (Stratagene), pKSXR (Fig 3.1). Subfragments of this plasmid (originally constructed by Dr Adam Rodaway), were isolated by enzymatic cleavage, and recloned into the same plasmid backbone (pKSXRa, pKSXRb, pKSXRc, pKSXRd). Sequence was initially generated by enzymatic synthesis from M13 primers complementary to the cloning vector, and later from internal primers based on derived sequence. Sequence obtained to the Nsi1 site at -497bp relative to the start site of transcription (Rodaway et al. 1990) was confirmed from each strand. Restriction enzyme recognition sites were determined by restriction mapping, and their presence confirmed in the derived sequence.

3.2.2 Base composition

Determination of base composition for the complete 3.2kb fragment indicates an over-representation of G and C nucleotides (48%) compared with total mammalian DNA (40%). The frequency of CG within the distal 350bp of the sequenced region (-2750 to -3100bp) is 10 times that predicted, and in comparison to GC dinucleotide frequency over the same region, suggests selective over-representation, and the existence of a CpG island. These findings are summarised (Fig 3.2).

3.2.3 Methylation pattern and DNAase1 hypersensitivity

To determine the methylation status of the CG rich region, genomic DNA extracted from both uninduced or retinoic acid-induced HL60 cells, and Daudi cells, was digested with Xmn1 together with either Msp1, or the methylation sensitive enzymes Hpa2 and Hha1. A Southern blot of the separated products was probed with a [^{32}P]-dCTP labelled DNA fragment extending from the Xmn1 to Nsi1 sites of pKSXR (Fig 3.2). In contrast to Msp1, and irrespective of the state of differentiation of HL60 cells, Hpa2 and Hha1 digestion products map to the region of CG over-representation (Fig 3.3).

Sequence	G+C	GC	CG
0 to -3100	48% (40%)	0.06 (0.04)	0.024 (0.008)
0 to -2750	48%	0.06	0.017
0 to -350	56%	0.06	0.029
-2750 to -3100	53%	0.10	0.080

(Bracketed figures represent expected frequency based on total mammalian DNA)

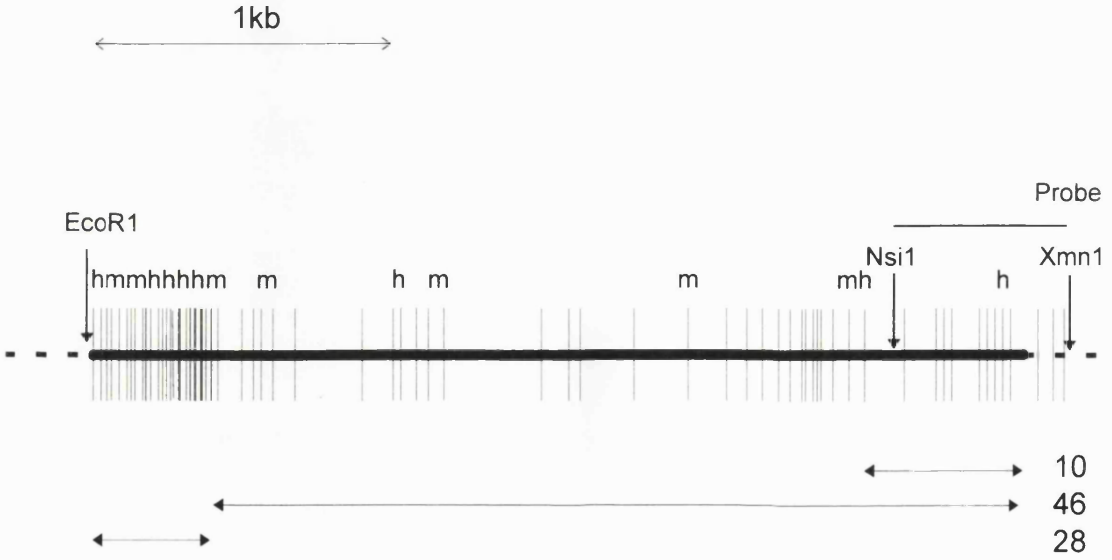


Fig 3.2. CpG dinucleotide repeat frequency. The table indicates the frequency of G and C single nucleotides (G+C),and the dinucleotides GC and CG in comparison with total mammalian DNA. The density of CG dinucleotides (represented by vertical bars) taken from the derived DNA sequence from the whole region is shown schematically. The total number for each region and enzyme recognition sites are also shown (m, Msp1/Hpa2 and h, Hha1). The probe used for DNAase1 and methylation studies is indicated.

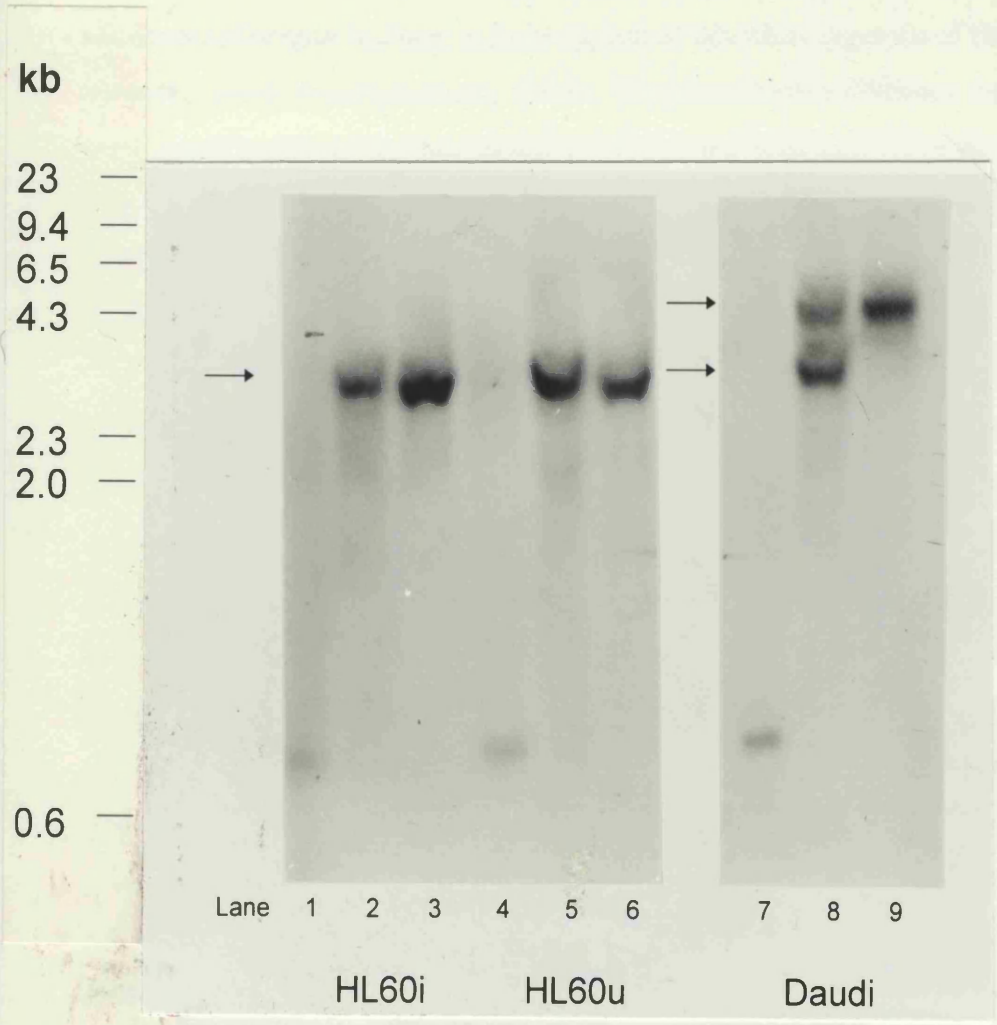


Fig 3.3. Methylation pattern of CpG-rich region. Genomic DNA from HL60 cells, either induced (HL60i), or uninduced (HL60u), and Daudi cells was digested with Xmn1 in addition to Msp1 (lanes 1,4,7), or the methylation sensitive enzymes HpaII (lanes 2,5,8) or Hha1 (lanes 3,6,9). In all cell types, products arising from digestion at unmethylated sites map to the CpG-rich region (3-3.5kb), and are indicated by the single arrow. Daudi cells show a partial methylation pattern at these sites, indicated by the release of a larger hybridising DNA fragment following digestion with HpaII and Hha1.

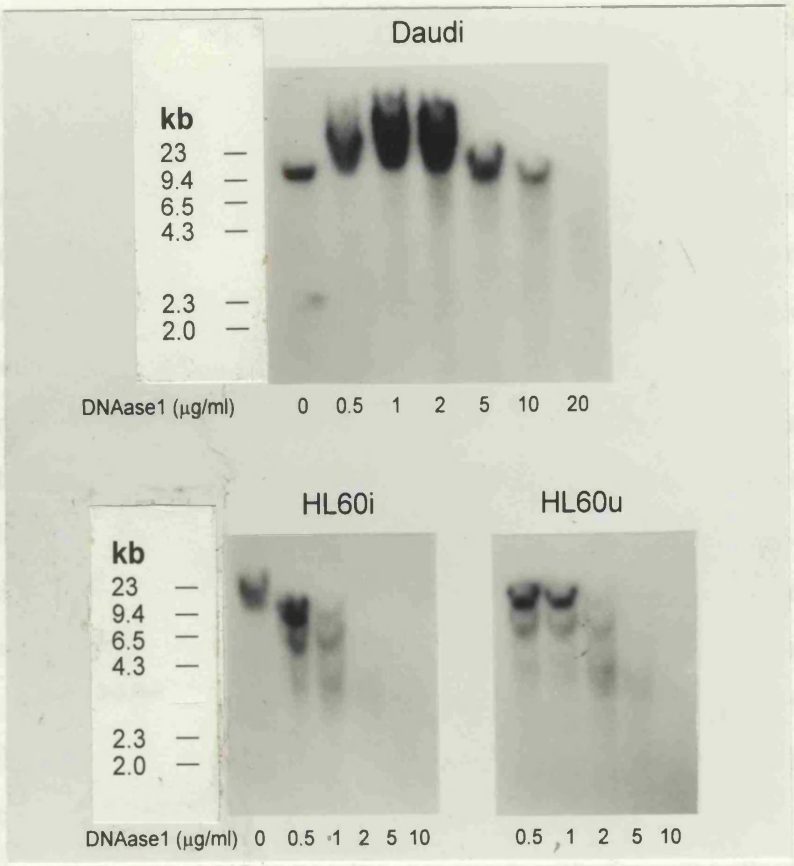


Fig 3.4. DNAase1 hypersensitive regions distant from the proximal promoter. Nuclei from HL60 cells, either induced (HL60i) or uninduced (HL60u), and Daudi cells were incubated with increasing concentrations of DNAase1 (0-20µg/ml), and extracted DNA digested with Xmn1. Separated products were transferred to nylon (Hybond N+), and probed with a labelled DNA fragment incorporating the proximal 500bp of the promoter. Hypersensitive sites, the most proximal of which co-incide with the CpG-rich region, are specific to HL60 cells, and more sensitive to DNAase1 in the induced cell type.

Restriction enzyme recognition sites consistent with the generation of these fragments were identified on the primary sequence (fig 3.2). In contrast, Hpa2 digestion of Daudi cell DNA released an additional larger fragment, suggesting partial methylation of the identified CpG-region in Daudi cells. In support of this, Hha1 digestion of Daudi DNA released a similar larger hybridising species. This may indicate a difference arising from tissue specific expression, but should be interpreted with caution in view of the tendency for cultured cells to undergo *de novo* methylation in cell-culture (Holliday and Ho, 1990). These observations suggest that the CG-rich region is undermethylated in HL60 cells, a characteristic which may be essential for regulated gene expression.

To determine the existence of expression-related DNAase1 hypersensitive regions upstream of the $p47^{phox}$ gene, intact nuclei derived from uninduced or retinoic acid-induced HL60 cells and Daudi cells were exposed to increasing concentrations of DNAase1. DNA was extracted, digested with Xmn1, and the products analysed by Southern blotting (Fig 3.4). Using the same probe as for methylation studies, increasing concentrations of DNAase1 revealed several new fragment species in both uninduced and induced HL60 cells including a doublet at approximately 3 to 3.5kb. Furthermore, DNA derived from induced HL60 cells would appear to be more sensitive than equivalent DNA extracted from uninduced cells, and many times more sensitive than Daudi DNA. Mature neutrophils, which are transcriptionally quiescent, are also relatively resistant to the effects of DNAase1 (not shown). These studies support the existence of a region of DNA approximately 3 to 3.5kb upstream of transcription initiation which may be functionally associated with tissue-specific and possibly differentiation specific expression of the $p47^{phox}$ gene. Other important regulatory regions may be situated further upstream, and are indicated by DNAase1 hypersensitivity sites which appear distal to the cloned CpG-rich region.

3.2.6 Repetitive DNA sequences

A large proportion of the total 3.1kb is made up of two regions of sequence derived from overlapping short interspersed sequences of repetitive DNA (SINES) belonging to the Alu family (Jelinek and Schmid, 1982). The first block, most proximal to the

transcription start site, occupies approximately 600bp and is arranged as a tandem repeat of two Alu sequences oriented in the direction of transcription (Fig 3.1). The first monomer of the second repeat is truncated, and is contiguous with a poly A tract from the second monomer of the first repeat, which itself appears to be complete. The second Alu region spanning approximately 850bp, comprises at least three, and maybe four overlapping repeats arranged in the same orientation. The average distribution of Alu sequence in human and Old World primate DNA is one dimeric repeat for every 5kb, indicating that this cloned region is particularly Alu sequence-rich. The significance of this observation is not clear. In addition to SINES, a region of simple sequence repetition is found at -1300bp, and consists of 19 tandem repeats of the consensus CATT. Allelic variation at this site has not been investigated.

3.2.7 Consensus sequences for transcription factor binding sites

DNA sequence from both strands of the promoter region extending from start of transcription to the Nsi1 site bordering the proximal Alu sequences, was scanned for recognisable transcription factor consensus binding sites (Fig 3.5). These are summarised (Fig 3.6). A non-classical TATA-box between –23 and –28 has a five out of seven base homology with the consensus (Breathnach and Chambon, 1981), and is probably sufficient to direct accurate initiation at the major transcription start site in the absence of GC-rich SP1-binding sequences, or homology to an initiator motif (weak consensus PyPyCAPyPyPyPyPy) (Smale and Baltimore, 1989). The absence of a CCAAT box is notable in view of mechanisms that regulate transcription of the gp91^{phox} gene. Other recognisable features include a possible binding motif for octamer-binding proteins, and three palindromic 'E-box' motifs (CANNTG) which may represent binding sites for helix-loop-helix (HLH) proteins. Three purine rich regions (PRS.1-3), one of which includes an exact match for the PU.1 DNA binding protein (GAGGAG), exhibit strong homology to interferon-stimulated response elements (ISRE), (LaMarco and McKnight, 1989), and have been implicated as important regulators of myeloid gene regulation (Fig 3.7). Between –280bp and –380bp, lies a complex region incorporating two repeated sequences. The first and third of these (Rep1 and Rep3) exhibit little homology with known binding-sites, but the second (Rep2) consists

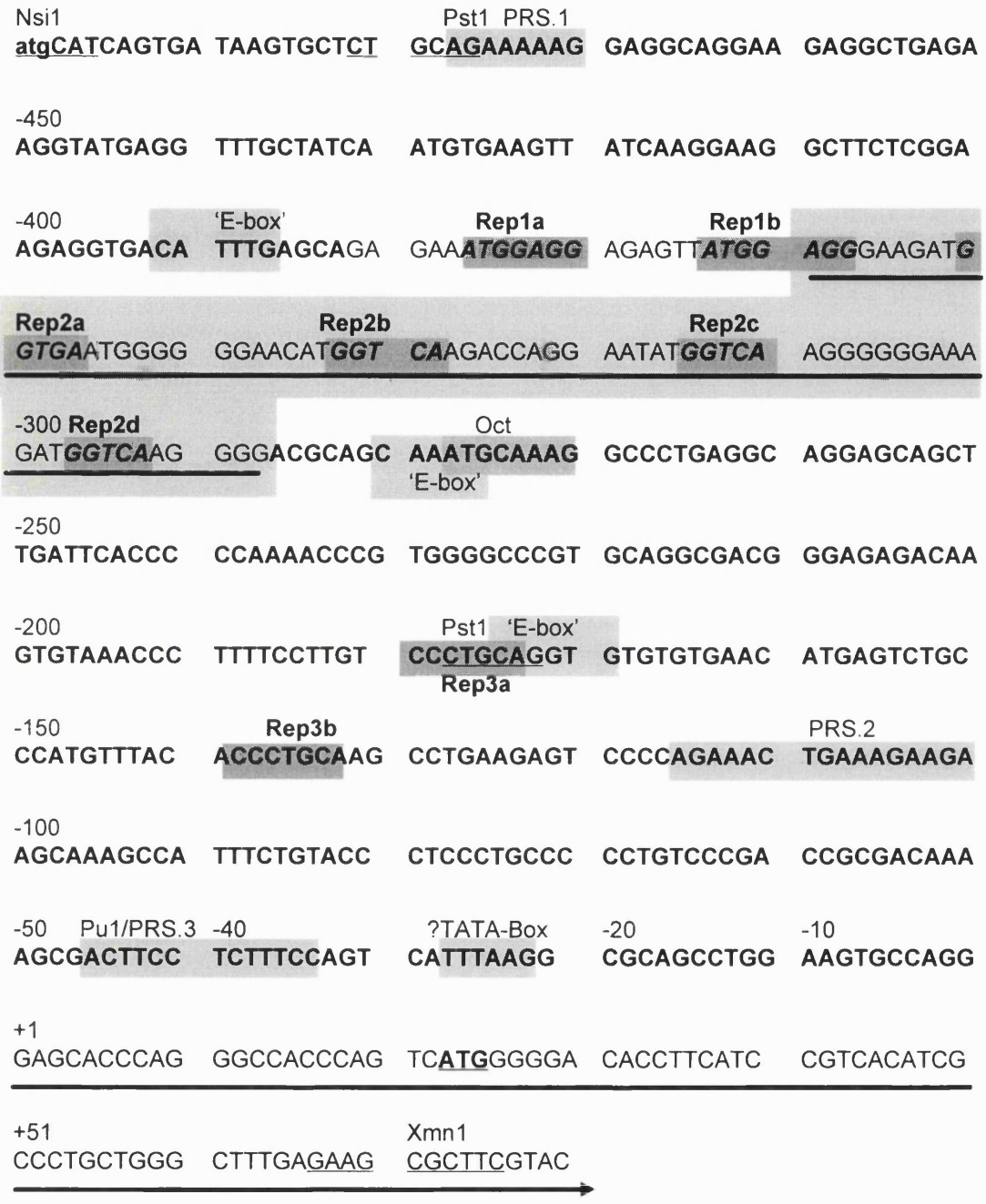


Fig 3.5. Primary sequence of p47^{phox} proximal promoter. Shaded sequence represents putative transcription factor binding sites for helix-loop-helix proteins (E-box), Octamer protein (Oct), Pu.1 protein (Pu.1) and TATA-binding protein. The transcribed region is indicated by an underlining arrow. Repetitive sequences are shown as Rep 1-3. PRS, purine-rich sequences. Restriction enzyme recognition sites are lightly underlined.

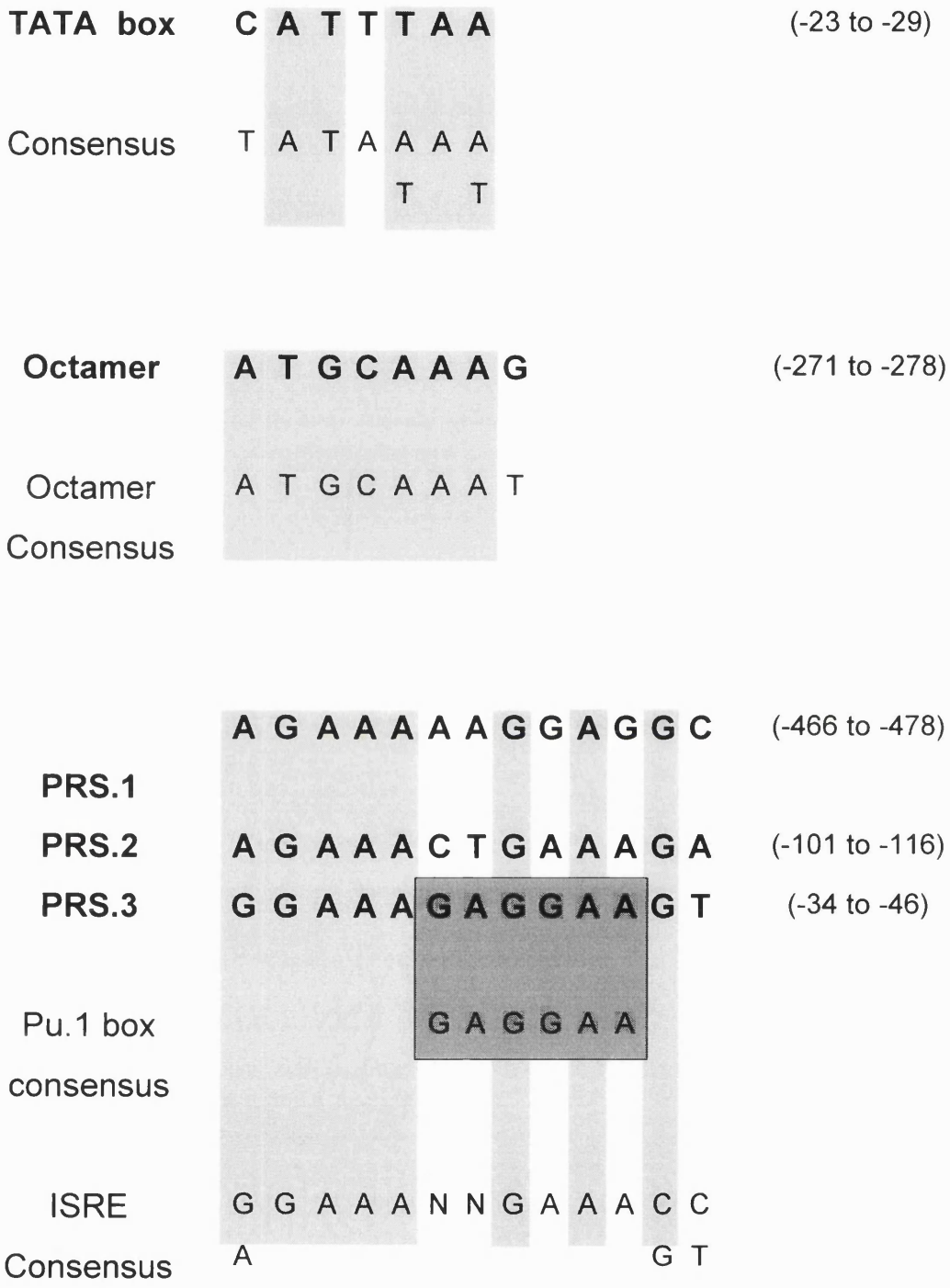
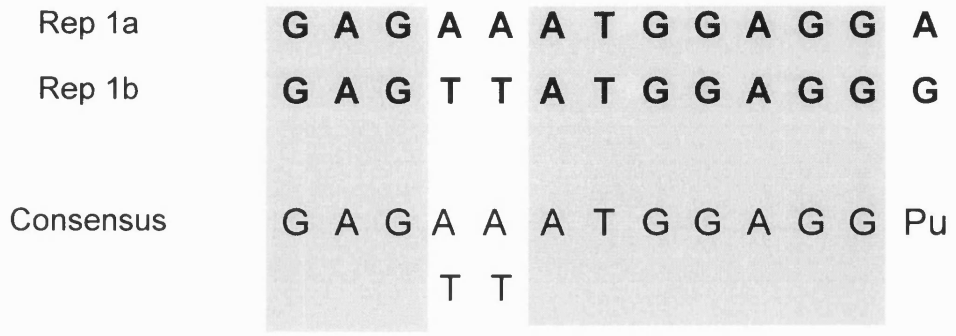
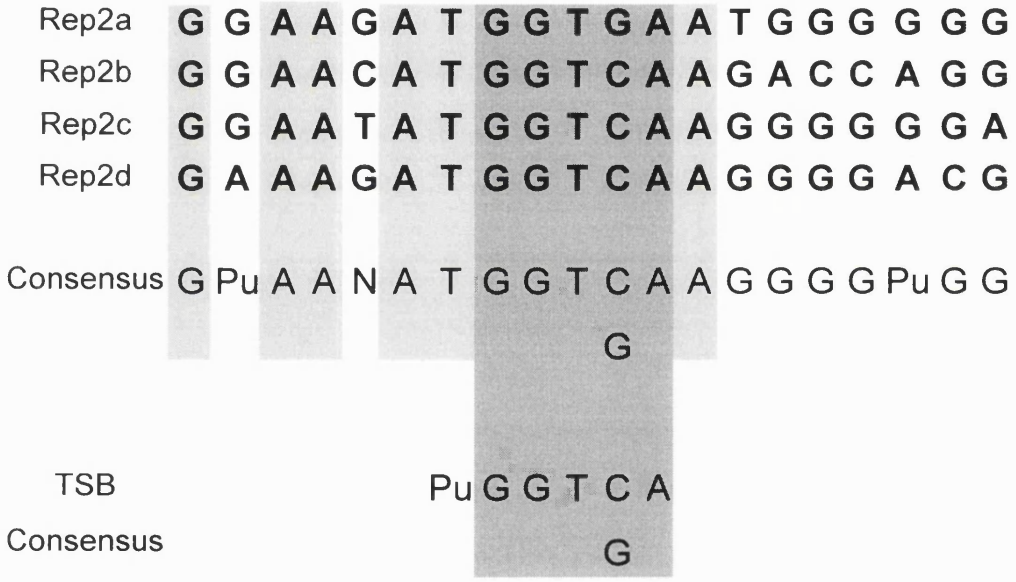


Fig 3.6. Putative transcription factor binding sites in p47^{phox} promoter. Consensus sequences are shown for a classical TATA-box, Octamer binding site, Pu.1 core binding motif, and an interferon stimulated response element (ISRE) aligned against homologous regions of the p47^{phox} promoter. PRS indicates purine rich sequence.

Repetitive element 1 (Rep 1 -358 to -377)



Repetitive element 2 (Rep 2 -288 to -353)



Repetitive element 3 (Rep 3 -133 to -174)

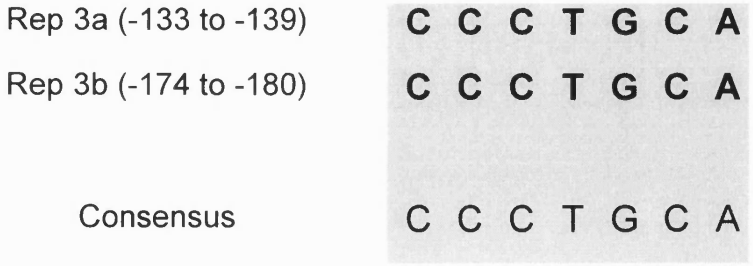


Fig 3.7. Repetitive elements within first 500bp of the p47^{phox} promoter. Each element is shown with a derived consensus. The heavily shaded area in Rep2 indicates homology with the binding site for the thyroid and steroid hormone family of transcription factors (TSB consensus).

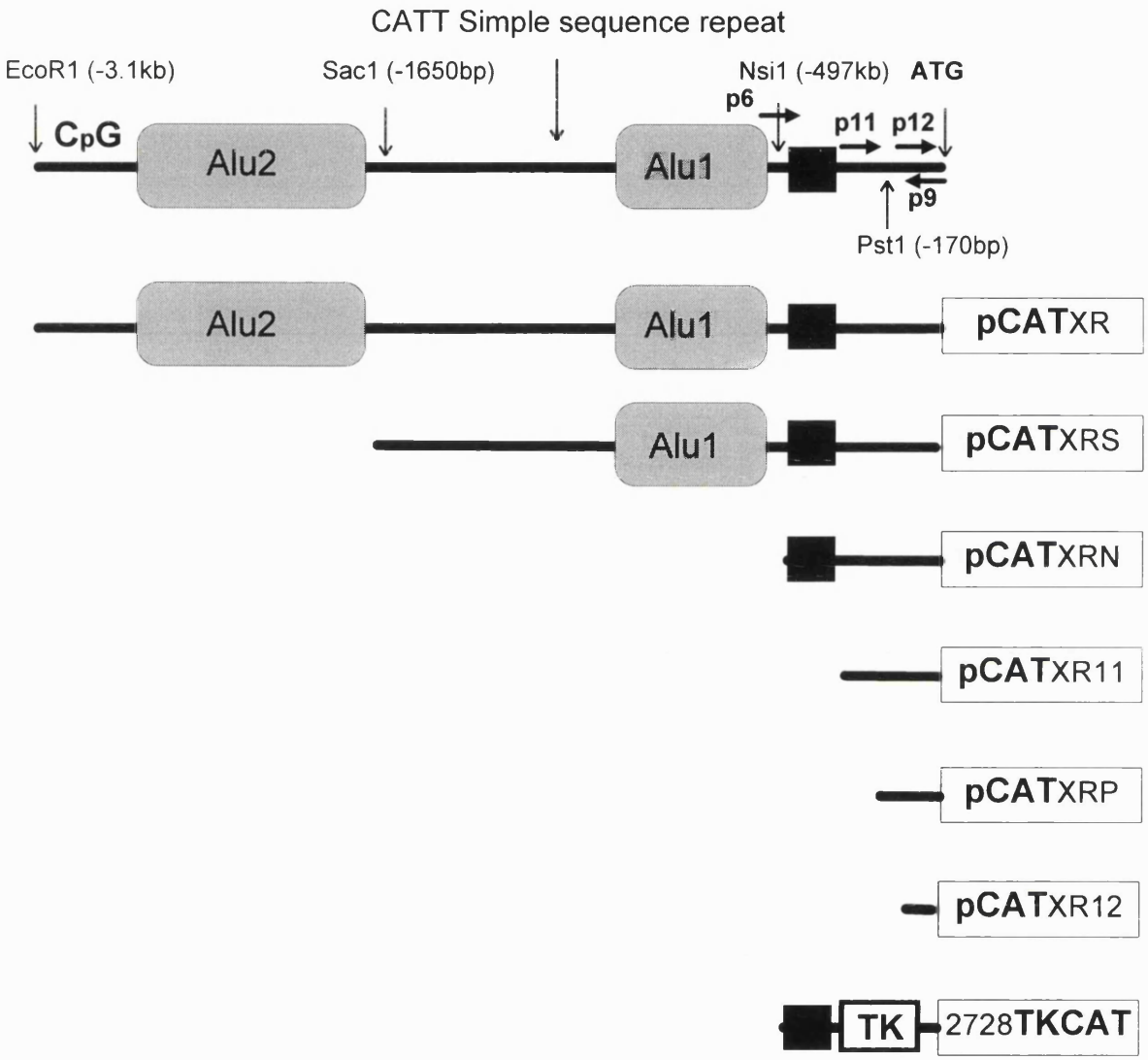
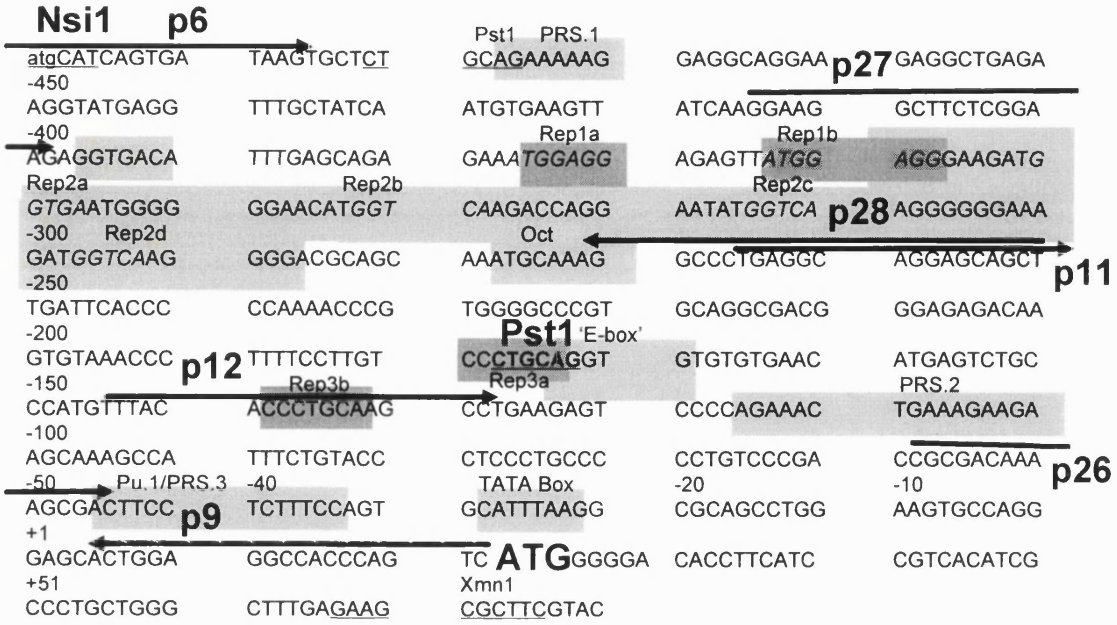


Fig 3.8. p47^{phox} promoter deletion series. Extending from -3.1kb (pCATXR) to -144 (pCATXR12), and linked to chloramphenicol acetyl transferase (CAT) reporter gene. Black box indicates a 165bp region spanning all four putative thyroid and steroid hormone receptor-binding half-sites, and TK a heterologous herpes simplex thymidine kinase promoter (-105 to +51 of mutant LS-115/-105). CpG represents CG dinucloetide rich sequence.

[**Construction of reporter gene vectors**: pKSXR was first modified by insertion of a 500bp PCR fragment extending from p47^{phox} start codon to the Nsi1 site (primers p9 and p6 incorporate Xba1 and Nsi1 recognition sites), effectively removing all coding sequence. The cloned PCR fragment was checked for errors by DNA sequencing. Digestion with EcoR1 and Sac1, Nsi1, or Pst1 followed by recircularisation produced pKSXRS, pKSXRN, and pKSXRP. Promoter fragments were excised and directionally cloned into HindIII and Xba1 sites of a promoterless CAT vector, pCATbasic (Promega), to form pCATXR, pCATXRS, pCATXRN, and pCATXRP]



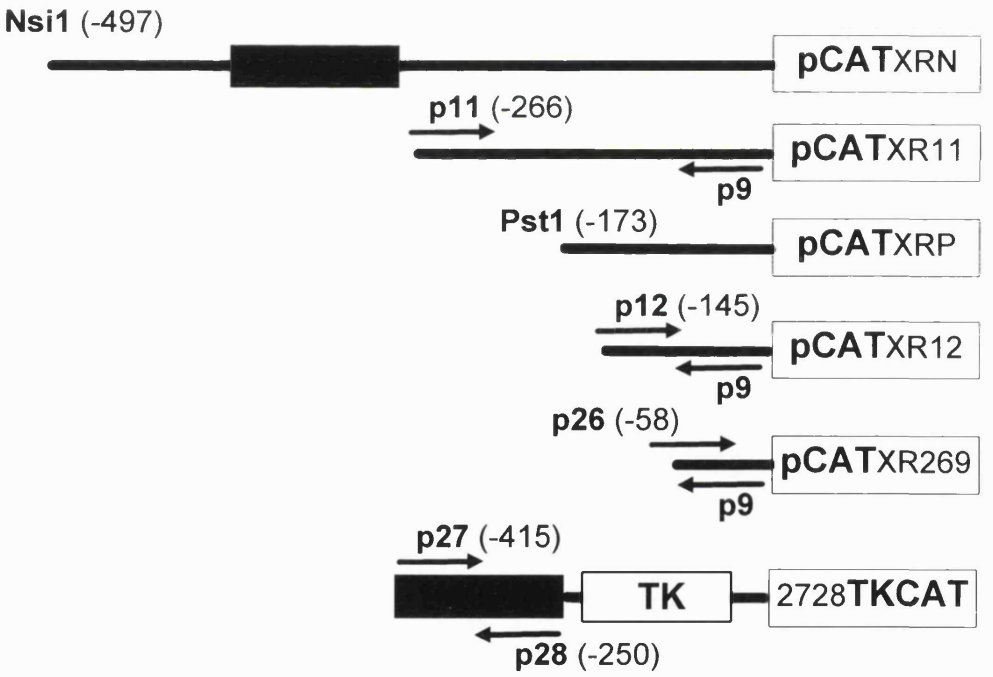


Fig 3.9. Sequence location of primers used for construction of vectors. Oligonucleotide primers (p6, p9, p11, p12, p26, p27, and p28, represented by arrows over sequence) and restriction sites used for construction of p47^{phox} promoter CAT vectors. Shaded sequence represents putative transcription factor binding sites as discussed in the text. TK is a heterologous herpes simplex thymidine kinase promoter.

[**Construction of reporter gene vectors**: PCR fragments generated from primers p11, p12, p26 and the complementary primer p9 were cloned into pCATbasic using HindIII and Xba1 restriction sites incorporated in the oligonucleotide sequence, to form pCATXR11, 12, and 269. 2728tkCAT was generated in a similar way from the reporter plasmid pBLCAT2. All PCR products were checked for errors by DNA sequencing]

of a unique arrangement of the thyroid and steroid hormone binding consensus (GGTG/CA, Parker, 1993) repeated four times, each element separated by exactly 13bp (Fig 3.7).

3.2.8 Functional characterisation of the p47^{phox} promoter

Functional elements within the cloned promoter region were investigated by transient reporter gene assay using the bacterial gene encoding chloramphenicol acetyl transferase (CAT). Construction of promoter-reporter gene vectors is summarised (Fig 3.8, Fig 3.9). To test activity, p47^{phox} promoter sequences extending from the endogenous initiation codon, and ranging between 58bp and 3.1kb in length were transfected into uninduced HL60 cells by electroporation. CAT synthesis was estimated by ELISA, and transfection efficiency controlled by colourimetric estimation of β-galactosidase activity expressed from a co-transfected vector (pSVβGal Promega). Results are shown (Fig 3.10[A]). Although all promoter fragments directed some CAT synthesis in comparison to the promoterless vector pCATbasic, this was generally low level. In contrast, expression of either LacZ or CAT from a SV40 early promoter was readily detectable. pCATXRN, which incorporates the proximal 500bp of the promoter consistently showed the highest activity, which was completely abrogated by removal of the distal 230bp, including both Rep1 and Rep2 sequences. The activity of the longest fragments pCATXR and pCATXRS, was diminished relative to pCATXRN, suggesting that sequences specific to the longer constructs direct inhibitory influences. In contrast, the smallest fragment pCATXR269, containing the PRS.3 and a putative TATA-box directed a 4-8 fold increase in levels of CAT expression over several experiments compared to pCATXR12, which in addition contains PRS.2.

Uninduced HL60 cells express low levels of p47^{phox}, and may therefore not support significant reporter gene activity. Steady state mRNA levels increase up to 50 fold on terminal differentiation to a mature phenotype. However, for cells transfected by any p47^{phox} promoter construct, a 48 hour period of induction by retinoic acid (RA, 1μM),

vitamin D3 (D3, 100nM), or DMSO (1.25% final concentration) failed to enhance activity. Efficacy of inducing agents was indicated by reduction in the rate of cell growth, increased adhesion to tissue culture plastic, and the ability to reduce NBT. Tissue-specificity of the $p47^{phox}$ promoter fragment which directed the highest level of activity in uninduced HL60 cells (pCATXRN) was tested by transfection of a number of different cell types (Fig 3.10[B]). Of the cells tested, activity in non-myeloid cells was negligible. In contrast, both uninduced HL60 and U937 cells exhibited significant activity over background, indicating some specificity of transcriptional activity.

As previously noted, within the distal 230bp of pCATXRN lies a complex region comprising four equally spaced repeats of the consensus GGTCA, although the distal repeat incorporates a G instead of C (GGTGA). This motif forms the binding-site for the thyroid and steroid hormone superfamily of transcriptional regulators (Beato, 1989). In view of published evidence for direct transcriptional activation of the $p47^{phox}$ gene by one member of the family, retinoic acid (RA), this region of the promoter is an obvious potential binding-site for cognate receptors (RAR). To explore this hypothesis, a 165bp fragment encompassing all four motifs was cloned in the same orientation upstream of a heterologous tk promoter, to form 2728tkCAT (Fig 3.9). This was first tested in uninduced HL60 cells, and was shown to direct more than 10-fold greater activity compared to the parent vector, indicating significant enhancer function (Fig 3.11[A]). However, when transfected cells were stimulated with RA, or another member of the same superfamily vitamin D3, further enhancement of activity could not be demonstrated. pCATXRN and pCATXRP were equally unable to induce CAT expression with either ligand, suggesting that the identified promoter region is unable to respond to either RAR or VDR (Fig 3.11[B]). Similar observations were made using the full length promoter fragment (pKSXR), and using thyroid hormone as a ligand (not shown).

* For all transfections, 20-30μg or 1-2μg of each plasmid was used per electroporation or calcium phosphate co-precipitation, respectively. Unless otherwise stated, transfection efficiency was determined by co-transfection of pSVβGal, and extracts normalised for this activity prior to CAT assay. All measurements of CAT synthesis fell within the limits of a linear calibration curve.

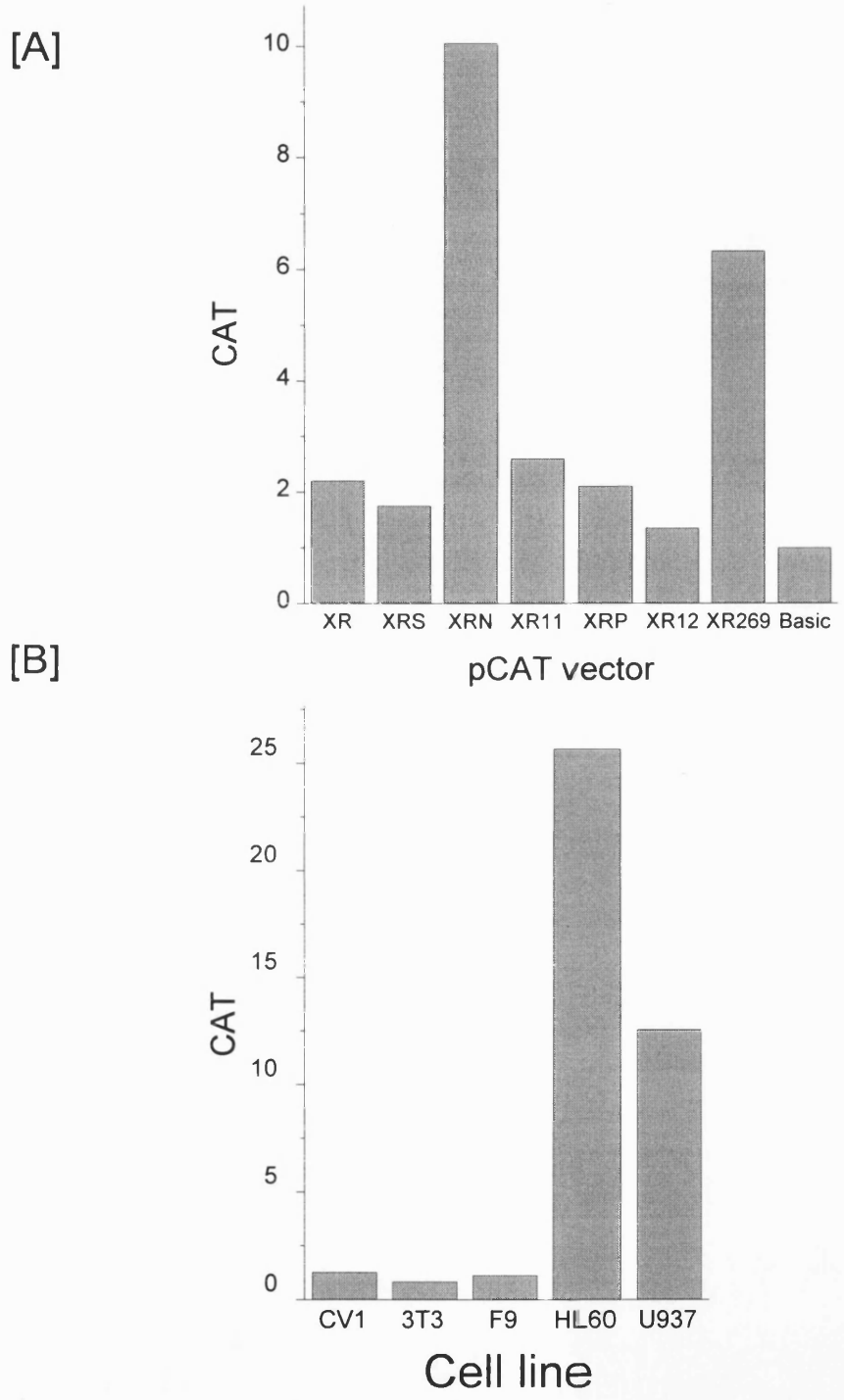


Fig 3.10. Promoter activity measured by reporter gene transfer*. [A] pCAT plasmid constructs containing fragments of the $p47^{phox}$ promoter were transfected into uninduced HL60 cells by electroporation, and synthesis of CAT measured after 48 hours by ELISA. Transfection efficiency was assessed by co-electroporation of the independent reporter plasmid pSVβGal (see materials and methods). CAT synthesis following electroporation of an equivalent plasmid incorporating an SV40 promoter was consistently 3-5 fold greater than that from pCATXRN (not shown). [B] Specificity of expression was tested for pCATXRN in CV1 (Green monkey kidney), 3T3 (murine fibroblast), F9 (murine embryonal teratocarcinoma), HL60 and U937 (human myeloid). Adherent cells were transfected by modified calcium phosphate co-precipitation, and suspension cells by electroporation (see materials and methods). CAT reporter gene expression is represented as fold synthesis of that obtained following transfection of the promoterless plasmid pCATBasic in each cell type. Results are representative of at least three independent experiments.

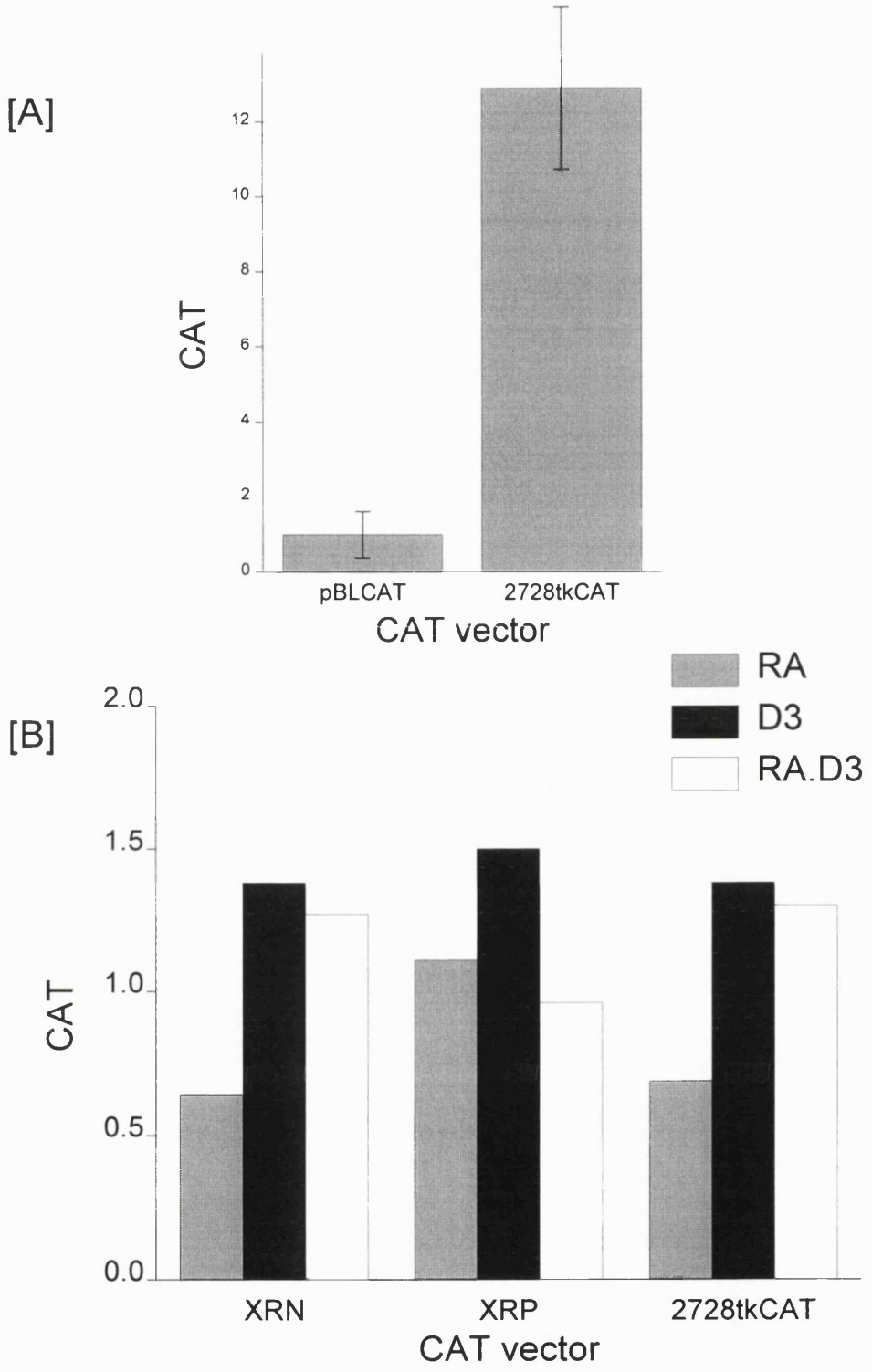


Fig 3.11. Promoter activity of nuclear hormone response element. [A] Enhancer activity of thyroid and steroid hormone response elements linked to a heterologous tk promoter (2728tkCAT) compared with the tk promoter alone (pBLCAT) in electroporated uninduced HL60 cells. [B] Response to induction of electroporated HL60 cells with retinoic acid (RA, 1μM), 1,25-di(OH) vitamin D3 (D3, 100nM), or both (RA.D3). For this experiment, cells were maintained in medium supplemented with 10% FCS stripped by ion exchange and charcoal adsorption. Cells were electroporated and after an overnight recovery in conditioned medium, split into four equal flasks, which were then left uninduced, or were induced with RA, D3 or both. Results are shown as fold-CAT synthesis over that in uninduced cells. The fragment containing the thyroid and steroid hormone response elements shows conspicuous enhancer activity in uninduced HL60 cells, but no evidence for RA or D3-mediated induction of expression could be found. Results are representative of at least three independent experiments.

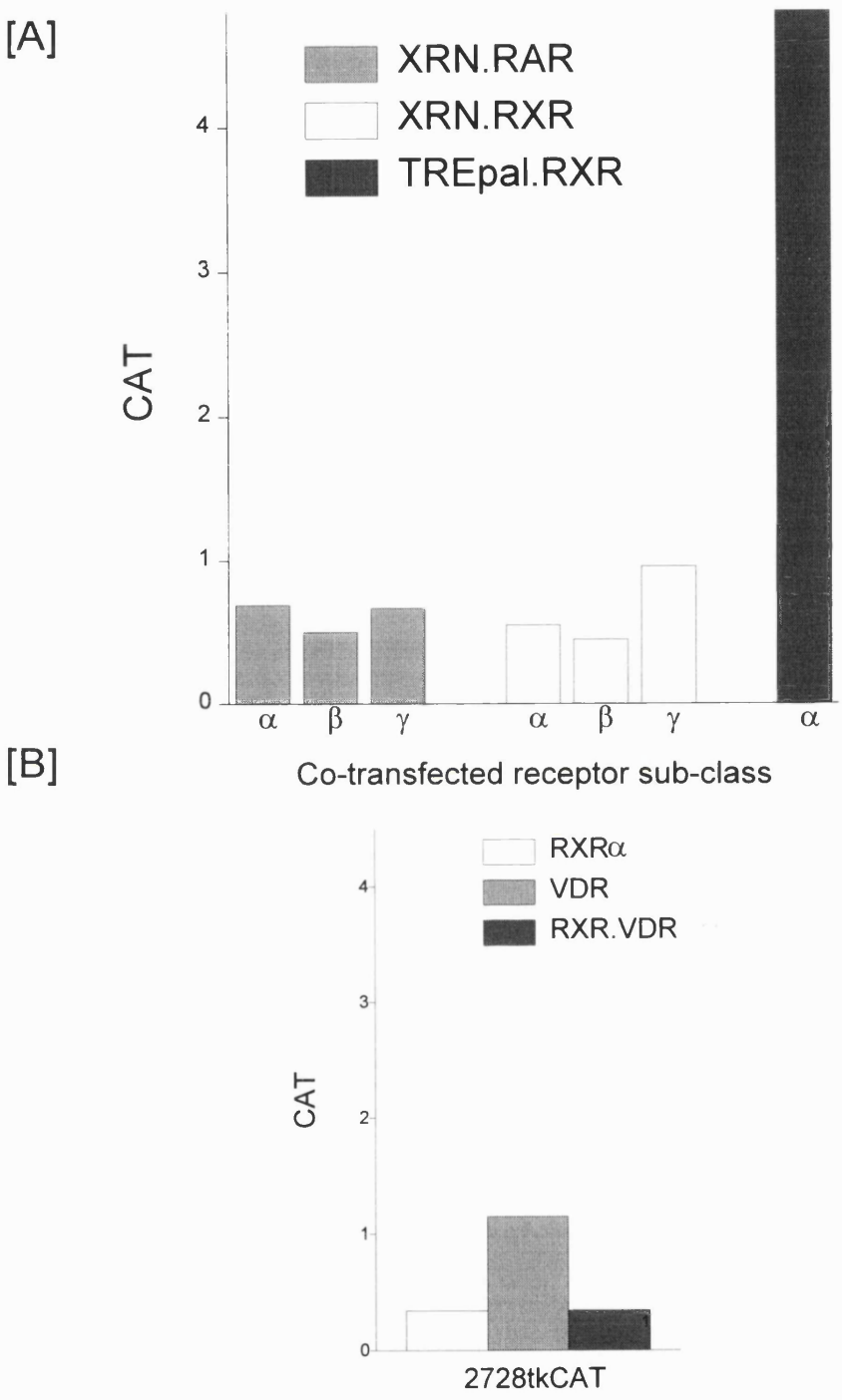


Fig 3.12. Activity of isolated nuclear hormone response elements. [A] pCATXRN was co-transfected with RAR or RXR expression plasmids (donated by P.Chambon) into CV1 cells by modified calcium phosphate co-precipitation. (Cells were maintained in DMEM supplemented with 10% FCS stripped of hormones by ion exchange and charcoal adsorption). RA (1μM) was added after an overnight transfection, and synthesis of CAT measured after 60 hours by ELISA. A synthetic thyroid and retinoid response element was used as a positive control. Transfection efficiency was normalised by measurement of β-galactosidase activity, expressed from a co-transfected reporter plasmid pSVβGal (Promega). Results are expressed as fold-levels of CAT synthesis compared to unstimulated cells. [B] 2728tkCAT was co-transfected as above into CV1 cells with RARα, RXRα or RXRα and VDR. After overnight transfection, cells were stimulated by addition of RA (1μM) or D3 (100nM). All results are representative of at least three experiments.

Background RAR/VDR activity in uninduced HL60 cells could be sufficient to obscure induced activity from this region. For this reason, both pCATXRN and 2728tkCAT were co-transfected with expression vectors encoding VDR, RAR and RXR α,β, and γ into an African Green Monkey kidney cell-line (CV1), which has negligible endogenous levels of these receptors. As before, no induction of activity could be observed by addition of either ligand (Fig 3.12A,B). Furthermore, activity was indistinguishable from that of non-transfected cells (not shown). In contrast, induction of activity from a CAT vector incorporating a palindromic thyroid hormone response element which also serves as an efficient RARE for RAR and RXR (TREpal), was significant. Similar results were obtained when 2728tkCAT and pCATXRN were transfected into mouse embryonal teratocarcinoma F9 cells, which endogenously express high levels of RAR. These studies were therefore unable to demonstrate that the repeated GGTC/GA motifs in the $p47^{phox}$ promoter form a functional binding site for either RAR or VDR.

3.3 DISCUSSION

3.3.1 Structure of the $p47^{phox}$ promoter

The distribution and frequency of CG dinucleotides in the first 3.1kb of the promoter are consistent with the existence of a CpG island probably extending beyond the boundaries of the cloned sequence. The frequency of CG over the whole region is 3 times that predicted for bulk DNA, but over the distal 350bp the frequency of CG approaches that of GC, and is 10 times predicted. Undermethylation is indicated by specific digestion at sites recognised by methyl-sensitive enzymes HpaII and Hha1 which map to this region. CpG islands are most commonly located at the 5' promoter regions of constitutively expressed housekeeping genes, but many examples exist in which expression of the associated gene is tissue-specific (Bird, 1986). CpG islands associated with tissue-specific genes are stably unmethylated irrespective of the state of gene expression in that tissue. For the $p47^{phox}$ gene, which is expressed predominantly in mature myeloid cells, the region of DNA with the highest frequency of CpG appears at least hypomethylated in both uninduced and induced HL60 cells, and partially methylated in

Daudi cells. Although undermethylation may be associated with tissue specific differences of gene expression, in the context of a stably unmethylated CpG island, this may also reflect *de novo* methylation (Holliday and Ho, 1990). Nearly all tissue-specific genes and one allele (sometimes both) of many housekeeping genes are inactivated by methylation in long-established cell lines. Approximately 50% of all gene inactivation events in cultured cells are the result of *de novo* methylation of affected genes at sites not methylated in normal tissues (Bestor and Coxon, 1993). The true significance of undermethylation in the $p47^{phox}$ gene promoter must be determined by analysis of DNA extracted from primary myeloid and non-myeloid tissues.

DNAase1 hypersensitivity sites map to regions between 3-3.5kb and approximately 6-8kb upstream of the transcription start site in both induced and uninduced HL60 cells, although induced cells are more sensitive. Both Daudi cells and neutrophils are relatively resistant. The proximal hypersensitive site maps to the region of high CpG density, and may indicate a mechanism by which local chromatin structure at these sites changes from 'closed' in those cells in which expression has ceased or is minimal, to 'open' during differentiation and maximal expression of the $p47^{phox}$ gene in myeloid cells.

The abundance of repeated elements within the promoter region is intriguing. Alu sequences (named because of a single Alu1 restriction enzyme cleavage site near the middle) are the most abundant family of middle repetitive sequences in the human genome, amounting to 3-6% of total DNA (Jelinek and Schmid, 1982). The 300bp human Alu sequences derive from a 130bp tandem duplication, and a 31bp insertion of unrelated sequence into the right half of the dimer. They are widely dispersed throughout the genome, and are flanked by heterogeneous short direct repeat sequences, resembling transposable elements. Individual members are not identical, but are related to 7SL RNA, an abundant small RNA necessary for translocation of newly synthesized proteins across membranes of the endoplasmic reticulum (Kariya et al. 1987). 7SL RNA is transcribed by RNA polymerase III, and apart from 155nt in the middle, is homologous to Alu sequence at both the 5'(90nt) and 3'(40nt) ends. Alu sequences therefore may have arisen from these genes. Some Alu sequences have been reported to

be transcribed from internal RNA polymerase III promoters *in vivo*, and when co-linear with protein coding sequences, by RNA polymerase II (Allan et al, 1983; Allan and Paul, 1984). It has been speculated that Alu sequences function as origins of DNA replication (Jelinek and Schmid, 1982), modulators of chromatin structure (Duncan et al. 1981), recombination hot-spots (Rogers, 1985), inhibitors of gene conversion (Hess et al. 1983) and mRNA stabilisers (Calabretta et al. 1981), although convincing evidence for most of these is lacking. For the $p47^{phox}$ promoter, Alu sequences are particularly abundant, at least within the first 3.1kb. The functional relevance of these sequences to transcription are obscure.

3.3.2 Identified transcription factor binding motifs

The basis for regulated myeloid expression of a number of genes outside the NADPH-oxidase complex has been characterised, but there is no emerging consensus of mechanism. In addition to the proto-oncogene transcription factors *fos* and *jun*, which are induced during normal myeloid differentiation (Lord et al, 1993), three members of the leucine zipper CCAAT/enhancer-binding protein family (C/EBP α,β,δ) are expressed at different stages of myeloid differentiation, suggesting important but undefined specific functions (Scott et al. 1992; Katz et al. 1993). CCAAT-displacement protein (CDP) has been implicated in repression of myelomonocytic expression of $gp91^{phox}$, but in the absence of a recognisable CCAAT motif in the $p47^{phox}$ promoter, is unlikely to be of importance for the regulation of this gene (Skalnik et al. 1991a). Helix-loop-helix (HLH) proteins have also been implicated in regulation of myeloid differentiation. The basic HLH (bHLH) sub-class of proteins recognise the palindromic 'E-box' motif, CANNTG (N, any nucleotide) (Blackwell and Weintraub, 1990). Some HLH proteins such as Id, Id-2, HLH462, and Emc (*Drosophila*) lack the basic amino acids necessary for DNA-binding, but inhibit the binding of other bHLH proteins. Consistent with a role in myeloid differentiation, constitutive expression of Id in an IL-3 myeloid cell-line blocked G-CSF-induced differentiation and 'E-box' binding activity (Kreider et al. 1992). Three 'E-box'motifs appear in the first 500bp of the $p47^{phox}$ promoter, and may therefore be important regulators of differentiation-linked expression.

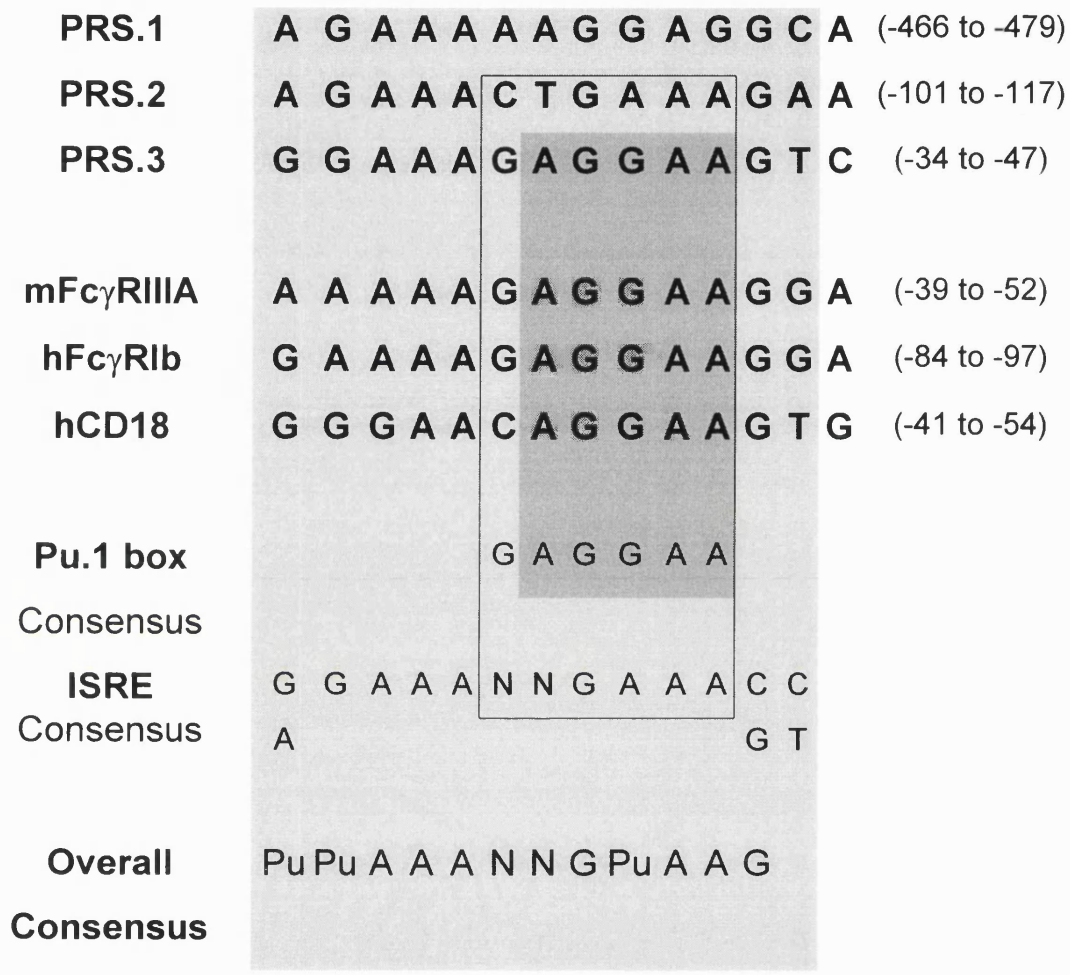


Fig 3.13. Comparison of purine-rich regions from mFcγRIIIA, hFcγRIb and hCD18 genes. Purine rich sequences from these genes have been shown to bind the transcription factor Pu.1, and are aligned with homologous sequences (PRS1-3) from the $p47^{phox}$ promoter, and an interferon-stimulated response element consensus (ISRE). Pu.1 has been implicated in both myeloid-restricted and Interferon-γ responsive gene expression.

Purine-rich sequences (Fig 3.13), of which three are found in the proximal 500bp of the $p47^{phox}$ promoter (PRS.1-3), are emerging as important regulators of myeloid gene expression, and have recently been implicated in the development of myeloid and lymphoid lineages from multipotential progenitors (Scott et al. 1994)). The purine-rich PU.1 *cis*-acting DNA element (GAGGAA) is found in numerous myeloid-specific promoters and has been implicated in the differentiation and activation of macrophages and B cells (Klemsz et al. 1990; Wasylyk et al 1993). PU.1 protein (identical to the *spi*-1 proto-oncogene) is a member of the *ets* family of DNA-binding proteins, and has been shown to regulate transcription of CD11a (Shelley et al, 1993), CD11b (Pahl et al. 1993), macrophage colony-stimulating factor receptor (Zhang et al. 1994), and macrophage inflammatory protein 1α (MIP-1α) (Grove and Plumb, 1993). Myelomonocytic expression of the murine low affinity Fc receptor IgG (FcγRIIIA), is directed by a 51bp region (–39bp to –90bp) designated the myeloid-restricted region (MRR), within which two cis-acting elements are recognised by PU.1 and bHLH proteins related to the ubiquitously expressed proteins TFE3 and USF, termed MyE (Feinman et al. 1994). In this context PU.1 is necessary, but not alone sufficient to direct myeloid specificity. Similarly, the myeloid-restricted pattern of expression of human high affinity FcγR1b is co-operatively mediated by a functional PU.1 site together with other necessary elements (Eichbaum et al. 1994). In this gene, which is specifically up-regulated by IFN-γ, a 181bp proximal promoter region contains a 39bp γ-IFN-response region (GRR, defined from the homologous FcγR1a gene), and a 21bp PIE motif in close proximity, which is conserved in the promoters of some other myeloid genes [**P**U.1 box (GAGGAA) and interferon responsive element, **IE** (RGAAAAG)]. In the context of the TATA-less promoter, this PU.1 box is essential for basal and IFN-γ inducible expression, and is hypothesized to bridge between the upstream IFN-γ enhancer, and an initiator sequence (Inr). In a separate study, GRR and PIE (designated myeloid cell-activating transcription element, MATE, in this study), formed an IFN-γ-activating complex (GIRE-BP) and a cell type-specific complex (MATE-BP) respectively (Perez et al, 1994). GIRE-BP is detected in many cell types after IFN-γ stimulation, but MATE-BP is specific to B cells and myeloid cells. Furthermore, MATE-BP (identified as PU.1), when expressed in non-haematopoietic cells, supported IFN-γ-induced expression of a reporter gene under the control of GRR and MATE,

indicating an important role for PU.1 in both tissue-specific and IFN-γ-inducible expression. Similarly, the minimal CD18 promoter which lacks both TATA, CAAT and initiator elements, consists primarily of *ets* repeats which confer tissue-specific and phorbol ester inducible expression (Bottinger et al, 1994).

Identification of three purine rich sequences in the proximal promoter region of the $p47^{phox}$ gene with strong homology to those of mFcγRIIIA, hFcγRIb (PIE or MATE), CD18, and to an ISRE consensus (LaMarco and McKnight, 1989), is likely to be significant (fig 3.13). PRS.3 in particular contains an exact PU.1 box, which in common with the CD18, CD11b, macrophage-colony stimulating factor receptor, mFcγRIIIA, and hFcγRIb promoters, is situated close to the start of transcription, and may therefore be an important determinant of tissue specificity. Interestingly, the proximal promoter region of the $gp91^{phox}$ gene contains two inverted purine-rich sequences, situated at similar locations to PRS.2 and PRS.3 relative to the TATA-box, which contain an ISRE consensus and a PU.1 consensus respectively (Skalnik, 1990a). Significantly, the smallest reporter gene construct which contains an exact PU.1 homology and the putative TATA-box directed several-fold higher levels of CAT expression in uninduced myeloid cells compared to a larger fragment containing PRS.2. This may indicate that the proximal PRS.3 acts as a bridge between upstream *cis*-acting elements and the TATA-box or initiation site in a mechanism similar to that proposed for the PU.1 region of the FcγR1b promoter, and may have important implications for myeloid and differentiation-specific expression of the $p47^{phox}$ gene. These regions are undergoing further evaluation. Direct transcriptional regulation by IFN-γ is not clearly established, but purine rich sequences such as those of PRS.2 and PRS.3, possibly in combination, are good candidates for functional response elements.

3.3.3 Thyroid and steroid hormone response elements

Evidence for direct transcriptional regulation of the $p47^{phox}$ gene by retinoic acid prompted the search for cognate binding sites (Rodaway et al, 1990). Retinoid receptors are members of the steroid receptor superfamily of proteins that function as ligand-dependent transcription factors, and includes steroids, thyroid hormones, and vitamin D3 (Green, 1993). Retinoic acid exerts its effects through two classes of receptor, the retinoic acid receptor (RAR), and retinoid X receptors (RXR). This classification is based on differences in primary structure, sensitivity to synthetic ligands, and ability to regulate expression of different target genes. Each receptor sub-family consists of several isoforms referred to as RAR or RXR α, β, and γ, suggesting that they are involved in different processes. The RAR family are activated by all-trans and 9-cis RA, whereas the RXR family are activated exclusively by 9-cis RA (Heyman et al, 1992). Unlike oestrogen and glucocorticoid receptors which form homodimers and recognise short palindromic DNA sequences, the vitamin D receptor (VDR), thyroid hormone receptor (TR), and RAR all bind to a direct repeat of the consensus GGTCA usually as a heterodimer with RXR (Beato, 1989; Umesono et al. 1991; Yu et al. 1991; Kliewer et al. 1992). The spacing of the direct repeat determines binding preference, and although it was originally proposed that each heterodimer acted through a unique direct repeat (VDR/RXR, 3bp; TR/RXR, 4bp; RAR/RXR, 5bp), there now appears to be considerable degeneracy (Naar et al, 1991).

Four repeated motifs GGTC/GA with exactly 13bp between each core consensus are therefore excellent candidate binding sites for members of the nuclear hormone superfamily. The arrangement of the repeats particularly resembles response elements for RA, D3 and thyroid hormone (T3). However, either in the context of the natural promoter, or a heterologous promoter, these elements were unable to confer RA, D3 or T3 inducibility to a reporter gene in myeloid cells. Similarly, in CV1 or F9 cells, co-transfection of RAR, RXR, or VDR receptors did not support induction of reporter gene expression. It is therefore unlikely that these motifs are RAR or VDR response elements, the implication being that the RARE lies outside the proximal 3.1kb of the promoter. Alternatively, the interpretation of previous run-off transcription data is incorrect, and the $p47^{phox}$ gene transcription is not directly inducible by retinoic acid.

One particular factor making interpretation of results more complex, is that terminal differentiation of HL60 cells is induced by RA, which itself causes widespread changes of gene expression.

In the absence of detectable induction by RA, D3 or T3, it is interesting to speculate on the function of this DNA sequence. Two features which are unique to these sites are the spacing between each presumed half site (13bp), and the consensus sequence TGGTC/GA. Almost invariably, the natural response elements for these hormones are based on the hexamer consensus PuGGTC/GA, the only example of a hexamer half site starting with T being the chicken vitellogenin A2 oestrogen response element (cVitERE, TGGTCA), which is arranged as a palindrome relative to its complementary half site (TGACCG) (Slater et al, 1991). Other members of the nuclear hormone receptor superfamily have now been described, many of which are termed 'orphan' because the ligand is unknown (Issemann and Green, 1990), and which bind as homodimers or as heterodimers with RXR. The repeated motifs in the $p47^{phox}$ promoter may act as a binding site for one of these, or for a unique member as yet undescribed. One candidate subfamily of orphan receptors are the chicken ovalbumin upstream promoter transcription factors (COUP-TF), which on the basis of amino acid sequence in the stem of the first zinc finger (P box), are related to TR and RAR (Wang et al, 1991). COUP-TFs have been found to activate promoters of a number of genes, and to down regulate hormonal induction of some genes by TR, RAR, and VDR (Cooney et al, 1991,1992,1993; Liu et al, 1993; Burbach et al. 1994). COUP-TF is relatively promiscuous for binding to diverse GGTCA repeat orientations and spacings, although binding affinity tends to diminish with increasing spacing, and one of 13bp has not been reported. Evidence for a COUP-like factor or factors binding to the $p47^{phox}$ promoter is based on weak enhancer activity in the context of both the natural and heterologous promoters. Furthermore, specific binding of a 38kDa protein to a 200bp fragment containing all four motifs can be competed by oligonucleotide sequences containing 2 half sites (Dr Colin Casimir, personal communication), and is reminiscent of the low molecular weight class of COUP-TF.

The arrangement of the four core GGTC/GA motifs is unique. The G-rich sequences after the first and third repeat particularly, suggest that each pair constitutes a functional binding site, and that intervening sequence for each pair in some way directs the conformation of DNA to enable specific binding to occur. The equality of spacing between each motif may signify a requirement for simultaneous occupancy and co-operativity at all four sites. Alternatively, these motifs may form 4 independent binding sites each with imperfect or palindromic second half sites, and would suggest that different receptors could bind within the same region (Fig 3.14).

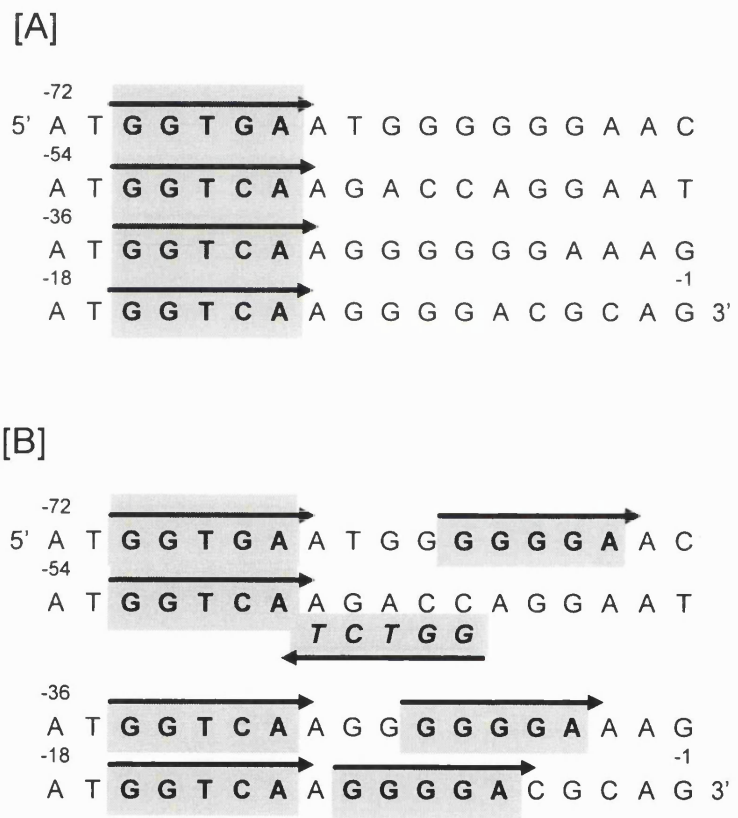


Fig 3.14. Speculative arrangement and orientation of hormone receptor binding elements. Equal spacing between each half-site suggests that they may co-operate as two pairs of binding sites [A]. Alternatively, each half-site may have a unique partner.

3.4 SUMMARY: PROMOTER ANALYSIS

- *The distribution and undermethylation of CpG dinucleotides indicates the existence of a CpG island which extends beyond the cloned region, and coincides with a site of DNAase1 hypersensitivity.*

- *The proximal promoter contains several potentially important transcription factor binding-sites, including PU.1, which is known to be an important regulator of expression for other myeloid-specific genes.*

- *The proximal 3.1kb promoter region of the $p47^{phox}$ promoter is insufficient to direct high level expression of a reporter gene in a transient assay system, inducible with terminal differentiation of immature myeloid cells. However, the proximal 500bp of the promoter directs low level tissue-specific expression of a reporter gene in myeloid cells.*

- *Four sequence motifs reminiscent of the nuclear hormone superfamily of binding elements together act as a weak enhancer, but are unable to direct ligand-inducible expression of a reporter gene in the context of either the natural or a heterologous basal promoter. The $p47^{phox}$ gene therefore may not be a target for transcriptional regulation by retinoic acid as previously described, or may be regulated by a binding element outside the tested promoter sequence. The cognate receptor(s) for the identified elements is(are) unknown, but could include a member of the COUP family of orphan receptors.*

CHAPTER 4:

RETROVIRUS-MEDIATED GENE TRANSFER

4.1 INTRODUCTION

The utility of both DNA and RNA tumour viruses as gene delivery vectors was first suggested by their ability to cause cellular transformation by transfer of genetic material. The most widely studied vector systems, and those which are currently the best developed for clinical use, are based on murine leukaemia retroviruses (MLV), members of a family of infectious agents, the Retroviridae, unified by common features of virion structure and replicative life-cycle (Coffin, 1992). Utilisation of these viruses as vectors necessitates comprehensive understanding of the natural biology of infection.

4.1.1 Structure of retroviral particles

Retroviral particles (virions) range from 80-130nm in diameter, and contain two identical copies of a plus-sense single stranded RNA genome complexed with viral-coded proteins from *gag* and *pol* genes. For the simplest retroviruses such as MLV, the genome is between 7 and 10kb in length. Retroviral virions also contain small RNA and DNA molecules which are probably incidental, and a specific tRNA unique to related family members, which primes RNA-dependant DNA synthesis. All retroviruses encode *gag*, *pro* (protease), *pol*, and *env* genes in this order, with *gag* and *gag-pro-pol* proteins synthesized from a full length mRNA species, and *env* protein synthesized from a spliced sub-genomic species (Fig 4.1). The nucleoprotein complex is surrounded by a host cell-derived lipid bilayer envelope which incorporates glycoprotein encoded by the *env* gene.

Retroviral *gag* genes encode polyproteins that are cleaved into at least three proteins designated matrix (MA), capsid (CA), and nucleocapsid (NC), a process which is mediated by products (PR) of the protease gene during virion assembly. Gag proteins constitute the major structural elements of the viral capsid. MA proteins line the inner surface of the envelope and are essential for membrane association and budding. CA

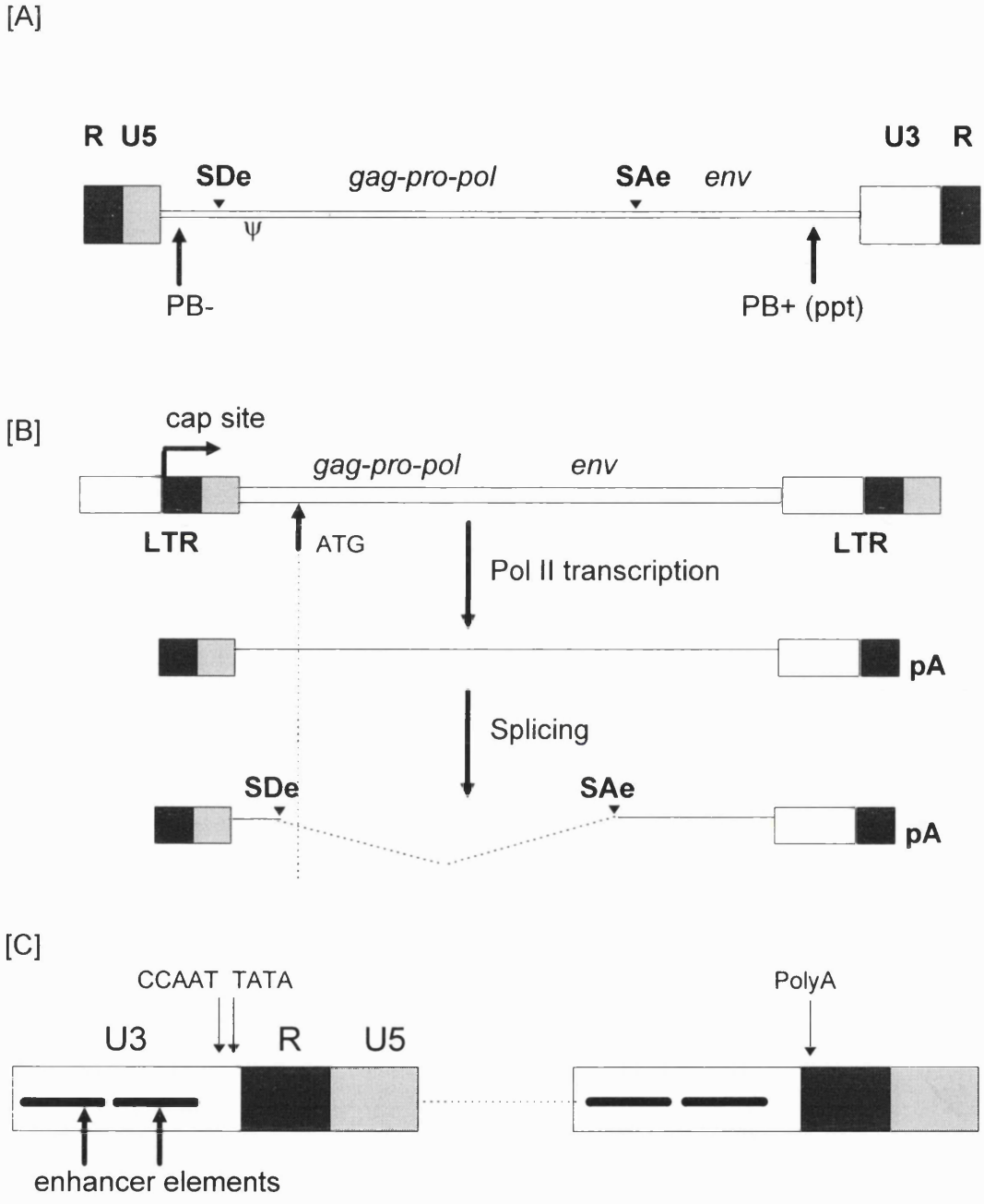


Fig 4.1. Retrovirus RNA genome and synthesis of full length and spliced MLV retroviral RNA. [A] Schematic of full length MLV RNA genome, showing replication primer binding sites (PB), splice donor and acceptor sites for *env* message (SDe, SAe). The position of the minimal packaging sequence is represented by ψ. The arrangement of the open reading frames are described in the text. [B],[C] The integrated MLV provirus acts as a template for transcription of viral RNA, which is mediated by cellular RNA polymerase II, and regulated by long terminal repeat sequences (LTR) formed during reverse transcription from the RNA genome. For MLV, polyadenylation is active in the 3' LTR but suppressed in the 5' LTR. The full length transcript is the precursor transcript for *gag-pro-pol* message, subgenomic *env* transcripts, and genomic RNA. For MLV, the *env* splice donor site (SDe) is upstream of gag initiation codon.

forms the core shell of the virion, and NC rests in intimate association with genomic RNA. In MLV, the *pro* gene is placed after the stop codon in *gag* but in the same reading frame as *pol*. Both *pro* and *pol* genes are expressed by in-frame readthrough facilitated by suppression of the termination codon at the end of *gag* by a glutamine tRNA. Reverse transcriptase (RT) is an RNA-dependent DNA polymerase encoded by *pol* which binds to the tRNA primer, and which in a distinct domain possesses RNaseH activity. Integrase (IN) is a separate protein derived from the carboxy terminus of the *pol* gene. The *env* gene encodes a polyprotein that is post-translationally modified by host cell enzymes to yield a transmembrane domain (TM), and an external glycosylated surface domain (SU) which determines the host range of the virus, and contains the major determinants for recognition by neutralising antibodies. TM anchors SU to the membrane, and mediates fusion of virus and host cell membranes. In membranes, SU and TM form complexes of 2-4 heterodimers each of which is stabilised by disulphide linkages (Avian sarcoma-leukosis virus, ASLV), or non covalent interaction (MLV).

The single stranded RNA genome consists of an internal region containing viral open reading frames, and flanking regions which are necessary in cis for replication of the virus (Fig 4.1A). The 5' end of virion RNA and viral mRNA is capped and methylated, and the 3' ends are polyadenylated. These modifications are undertaken by host cell enzymes. A short repeated sequence R is located at each end of the genome and mediates DNA strand transfer during reverse transcription. Another short sequence U5, is located between R and the minus-strand primer-binding site (PB-) at the 5' end of the genome, and is necessary for formation of secondary structures which guide efficient initiation of reverse transcription. PB- is complementary to 16-19 bases at the 3' end of the specific tRNA molecule. Following PB is an untranslated leader sequence which precedes the initiation codon for *gag* and which incorporates a packaging sequence (ψ) which for MLV is about 300bases long, and lies immediately downstream from the *env* splice donor site (SDe). Subgenomic *env* mRNA is therefore not assembled into virions because the cis-acting signal is in the *env* intron (Fig 4.1B). At the 3' end of the genome, downstream of *env* a short purine rich sequence (polypurine tract, ppt) initiates synthesis of plus-strand DNA (PB+). U3 is unique to the 3'end of the RNA genome, but

is duplicated in the proviral genome, and contains elements necessary for transcriptional regulation of the integrated provirus (Fig 4.1C).

4.1.2 Retroviral lifecycle

Viral host range is primarily determined by interaction between the SU domain of *env* and the cell-surface receptor, although intracellular factors may influence replication (Luciw and Leung, 1992). Type C MLV can be divided into four host range or interference classes which differ predominantly in the *env* glycoprotein, the most divergent regions of which are found in the amino-terminal half of SU (Steck and Rubin, 1966; Morgan et al. 1993). Ecotropic viruses infect only rodent cells, and amphotropic viruses infect cells from many species. Xenotropic viruses infect non-murine cells, but are generally unable to infect murine cells. Polytropic viruses are products of recombination events between ecotropic viruses and endogenous murine proviral sequences, and can infect most cell types. Seven cell surface receptors for retroviruses have now been identified, including those for human and simian immunodeficiency viruses (CD4), avian leukosis virus type A (Tva1), bovine leukaemia virus (Blvr), feline immunodeficiency virus (CD9), ecotropic viruses (MCAT) (Albritton et al. 1989), amphotropic viruses (Ram-1) (D.G.Miller et al. 1994, van Zeijl et al. 1994), and a common receptor (Glvr-1) for gibbon ape leukaemia virus (O'Hara et al. 1990), feline leukaemia virus group B and simian sarcoma-associated virus. MCAT is a cationic amino acid transporter, and Glvr-1 and Ram-1 are phosphate transporters (Kavanaugh et al. 1994). Following binding, virus and cell membranes fuse, a process mediated by the TM domain of *env*, and the virion capsid is released into the cytosol. Viral enzymes RT and IN remain associated with genomic RNA in a nucleoprotein complex, within which reverse transcription and generation of a linear double stranded DNA with long terminal repeats (LTR) take place in the cytosol. The model for reverse transcription is shown (Fig 4.2).

Following reverse transcription, the nucleoprotein complex which incorporates the blunt ended DNA molecule, gains access to the nucleus, and is integrated into host cell DNA. For many retroviruses including MLV, entry of the preintegration complex

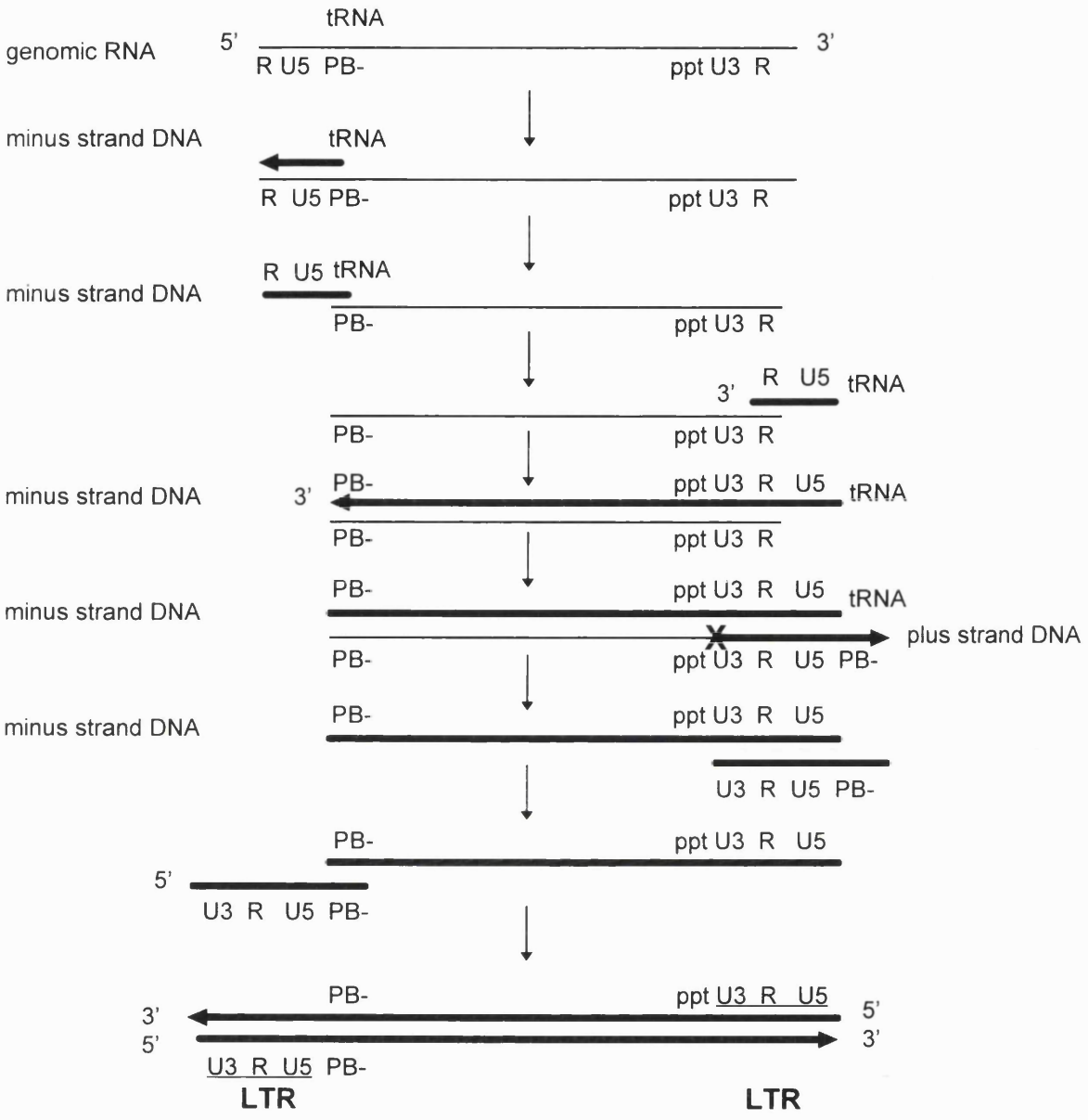


Fig 4.2. Model for reverse transcription of retroviral genome. Reverse transcription is primed by a specific tRNA molecule by base-pairing at the primer binding site (PB-). The R/U5 template is partially or totally degraded by RNaseH activity of reverse transcriptase. This allows complementary sequences from the minus strand strong stop DNA copy to base pair with the R sequence of the 3' end of the RNA molecule by both *intra-* and *inter*-strand transfer, and continue synthesis. RNaseH degrades the genomic RNA of the hybrid molecule, leaving an oligonucleotide primer at the polypurine tract (ppt), for initiation of plus strand DNA synthesis (**X**). A second *intra-*molecular strand transfer is followed by elongation and generation of full length blunt-ended double-stranded DNA, with duplicated long terminal repeats (LTR). Bold lines indicate DNA.

to the nucleus is dependent on the cell passing through mitosis, at which time the nuclear membrane is disrupted (Roe et al. 1993). Proviral integration mediated by IN protein which has site-specific nuclease activity, occurs in post-replication DNA (Craigie et al. 1990; Hajihosseini et al. 1993). The integrated provirus is flanked by direct repeats of 4-6bp in the host DNA, and itself ends with short inverted repeats. In contrast, the lentivirus HIV possesses specific nuclear localisation sequences within MA, which result in active translocation of the preintegration complex to the cell nucleus, and establishment of an integrated provirus independent of cell mitosis (Bukrisky et al 1993). Studies on proviruses in infected cells show that they are found more frequently in actively transcribed regions and sites of DNAase1 hypersensitivity (Whitcomb and Hughes, 1992). The late phase of retroviral replication includes LTR-directed synthesis and processing of viral RNA, and assembly of immature virus particles. Budding through the cell membrane results in the acquisition of a lipid bilayer which incorporates *env* glycoprotein.

4.1.3 Regulation of transcription by LTR sequences

For simple retroviruses such as MLV, LTR sequences contain most cis-acting elements necessary for transcription initiation and processing (fig 4.1c). The retroviral cap site defines the 5' end of the R region. Regulation of transcription is controlled by sequences including a TATA box within the U3 region. MLV enhancer elements are located in two 75bp tandem repeats approximately 150bp upstream of the TATA box, and by promoter insertion are partly responsible for the slowly transforming characteristics of this retroviruses which does not itself encode an oncogene. Tissue specificity of expression dictates the pattern of tumour induction, and is largely controlled by enhancer elements, although sequences flanking the enhancer elements may be influential. Moloney MLV (MoMLV) enhancer sequences function efficiently in T-lymphoid cells and cause T cell lymphoma, whereas those of Friend-MLV (F-MLV) function preferentially in erythroid cells and induce erythroleukaemia. Exchange of core elements within the enhancers exchanges tumour specificity, but is not dependent on a single core element (Golemis et al. 1989; Hollon and Yoshimura, 1989). Mutant MoMLV, which lacks a 23bp GC-rich region flanking the enhancer, produces a wider range of tissue tumour types (Hanecak et al. 1991).

Provirus expression is variable between different cell types, and most notably is repressed in embryonal carcinoma cells (EC) and stem cells (ES). In contrast, levels of expression in differentiated cells such as 3T3 fibroblasts are high. Low levels of MoMLV expression in EC cells may be in part a result of a deficiency of positive acting factors (Speck and Baltimore, 1987), but is predominantly due to activity of *cis*-acting repressor sites in the genome (Gautsch and Wilson, 1983; Barklis et al. 1986; Weiher et al. 1987). One of these sites is termed the upstream conserved region (UCR, conserved in over 90% of mammalian type C retroviruses) and binds the ubiquitous factor transcription factor YY1 (Flanagan et al. 1992). Repression has also been shown to be mediated through a repressor binding site (RBS) which overlaps the primer binding site (PB) region (Kempler et al. 1993), and may either directly or indirectly be associated with methylation of the provirus genome (Stewart et al. 1982; Hoeben et al. 1991).

4.2 RETROVIRUS-BASED VECTOR SYSTEMS

The natural life cycle of retroviruses has been exploited for highly efficient and stable transduction of heterologous genes. The major advantages of retroviral vectors are high efficiency gene transfer to replicating cells, and stable integration of genes into host DNA at low copy number. Furthermore, retroviral vectors are generally replication defective and therefore limited to one round of infection only. Host range is determined largely by *env* gene products which is therefore a potential site for modification. The majority of retroviral vector systems for mammalian cells are based on MoMLV, and consist of two components, the vector containing heterologous genetic material, and a modified retroviral genome from which protein components of the retroviral particle are expressed.

4.2.1 Retroviral vector packaging cell lines

The most significant advance in the development of retroviral vector systems was the development of packaging cell lines. These are engineered to produce all trans-acting factors necessary for production of retroviral particles. The first generation of packaging cells incorporated a MoMLV provirus from which the packaging site ψ had been deleted. These cell lines based on murine 3T3 cells (ψ-2 (ecotropic envelope) and ψ-am

(amphotropic envelope)) transcribed all mRNA species necessary for synthesis of *gag pro-pol* and *env* proteins but were unable to package this genome (Mann et al. 1983; Cone and Mulligan, 1984; Miller et al. 1985). In contrast, defective retroviral vectors encoding reporter genes which retained the ψ sequence were packaged into infectious particles, and because they lacked genes necessary for retroviral replication, were limited to one round of infection.

However, a single recombination event between vector and packaging genome could result in production of replication competent virus, and further modifications have been made to the packaging sequences which reduce the frequency of this occurrence, yet retain the ability to produce high titres of recombinant virus. In the ecotropic cell line PE501, and the amphotropic equivalent PA317, the recombinant retroviral genome has deletions in the 5' LTR, and a heterologous SV40 polyadenylation signal in place of the 3'LTR, in addition to a deleted ψ sequence (Miller and Buttimore, 1986). More sophisticated packaging cell lines further reduce the chances for generation of replication competent 'helper' virus by expression of *gag* and *pol* from one construct, and *env* from another (ψCRE and ψCRIP (Danos and Mulligan, 1988), GP+E86 and GP+AM12 (Fig 4.3) (Markowitz et al. 1988), ΩE (Morgenstern and Land, 1990)). ψCRE and ψCRIP constructs are designed with reciprocal linker insertion mutations that destroy either *gag* or *env* open reading frames, so that for both constructs there is a large sequence overlap in the *gag-pro-pol* region. In contrast, GP+E86/GP+AM12 and ΩE have extensive reciprocal deletions so that for GP+E86 and GP+AM12 overlap is less than 500bp, and for ΩE, less 66bp. Furthermore, homology within this 66bp region is reduced to 55% by codon wobble. Sequential transfection with different selectable markers transferred ψCRE/ψCRIP and GP+AM12 packaging constructs into cells, whereas GP+E86 and ΩE were co-transfected with a single marker.

4.2.2 Retroviral vectors

Retroviral vectors in which heterologous genetic material was inserted into the backbone of MoMLV were first developed in the early 1980's (Tabin et al. 1982).

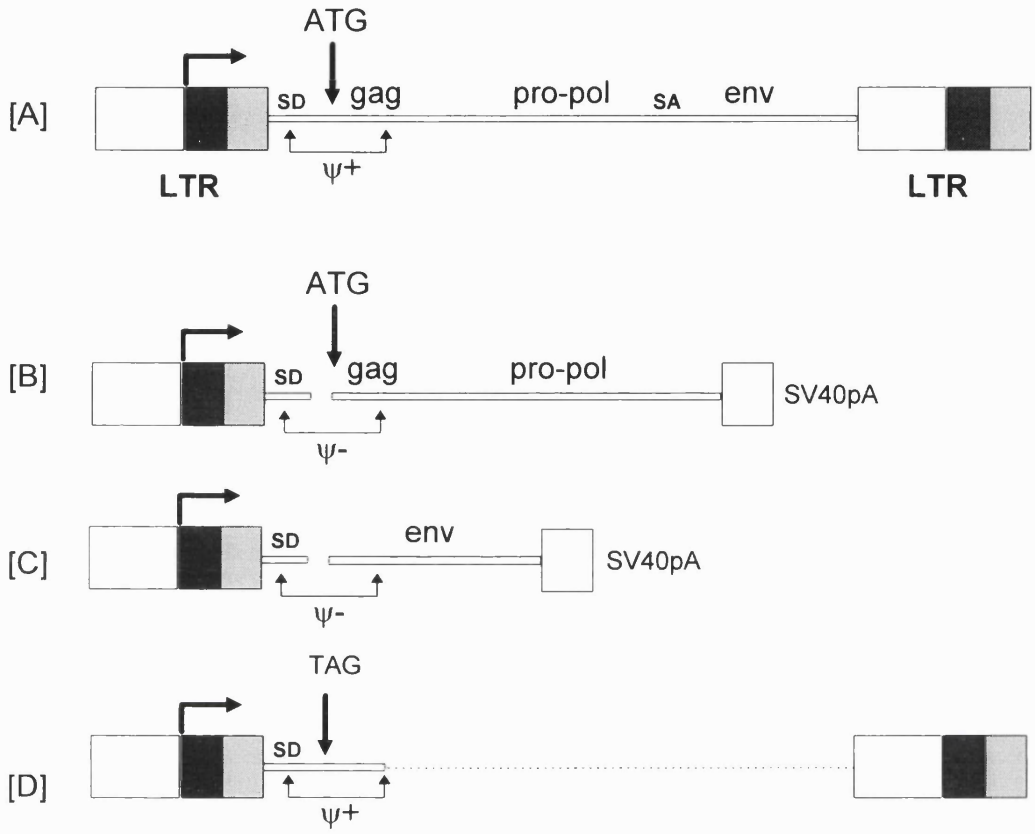


Fig 4.3. Retroviral packaging system (Markowitz et al. 1988). [A] Wild-type MoMLV 9.2kb genome in which ψ+ denotes the extended packaging sequence. [B],[C] GP+E86 and GP+AM12 constructs. *gag-pol* and *env* functions are situated on different plasmid constructs, which in both cases are ψ-deleted, and have had the 3' LTR replaced by an SV40 polyadenylation sequence. [D] Basic retroviral vector in which the ATG for *gag* is mutated to TAG, and which incorporates the extended packaging sequence.

Later, cis-acting encapsidation signals (ψ) in the leader sequence of the MLV genome defined minimum sequences that were necessary for rescue of recombinant vector genomes (Mann et al. 1983; Cepko et al. 1984), and permitted removal of *gag pol* and *env* coding regions. Although not absolutely determined, up to 8kb of heterologous material can be incorporated. Significant increase (up to 40-fold) in the efficiency of rescue followed when part of the 5' *gag* sequence was included with ψ, which together is designated ψ+ (fig 4.3d) (Adam and Miller, 1988). Mutational inactivation of the unpaired SD site upstream of *gag* has also been reported to enhance levels of expression. Retroviral vectors may express single or multiple genes. Two genes, one of which is often a drug resistance gene, can be expressed using the natural splicing mechanism of the virus, or using two promoters (Cepko et al. 1984). In this latter example, the drug resistance gene is usually transcribed from an internal promoter, while the therapeutic gene utilises the LTR. However selection for transferred drug resistance does not necessarily select for optimal expression of the therapeutic gene. Incorporation of an internal ribosome binding site (dicistronic vectors) may overcome this problem, because both gene products are translated from the same message. Similarly, when expression of a heterologous gene is regulated by an internal promoter, LTR influences can be inhibitory (Emerman and Temin, 1984, 1986). One approach to overcome this is inactivation of the LTR. In self-inactivating vectors (SIN), the 3' U3 LTR domain of the vector construct is replaced by a heterologous promoter (Yu et al. 1986). Following reverse transcription and integration, both LTRs of the provirus will be deleted for U3 and contain only the heterologous promoter sequences. Unfortunately, SIN vectors often produce relatively low viral titres. In most cases, vectors in which a single gene is transcribed from the retroviral LTR produce both highest titre, and most consistent expression. One of the most widely used current vectors (MFG), contains a single gene inserted as close as possible to the natural *env* translation start site, and retains the natural *env* splice donor and acceptor sites. This may enhance the stability of message, and efficiency of translation.

4.3 RETROVIRUS-MEDIATED P47^{PHOX} GENE TRANSFER

The ultimate target cell population for curative gene therapy of CGD is the pluripotent haematopoietic stem cell (PHSC), which is defined by a capacity for extensive self-renewal and retention of multilineage differentiation potential. The following preliminary experiments were designed to test functional correction of biochemical phenotype in an intact cellular model of p47^{phox}-deficient CGD, and subsequently to demonstrate successful gene transfer to bone marrow progenitor cells.

4.3.1 Measurement of NADPH-oxidase function in immortalised B cells

Primary phagocytic cells are resistant to retrovirus mediated gene transfer for reasons outlined previously. EBV-immortalised B lymphocytes express all components of the NADPH-oxidase, and produce measurable quantities of superoxide following stimulation by agents such as PMA. Cells derived from patients with CGD mirror the functional defect of primary phagocytic cells, and are therefore useful models for reconstitution of the NADPH-oxidase activity by gene transfer.

The most sensitive and specific methods for detection of NADPH-oxidase activity are based on chemiluminescence. SOD-inhibitable direct reduction of ferricytochrome c has the advantage of being quantitative, but is less sensitive, and can be misleading at low levels of activity. For immortalised B cells, which at best produce a small percentage of the superoxide generated by phagocytic cells, detection methods based on chemiluminescence are preferable. Similarly, because existing methods for cell-free determination of NADPH-oxidase activity were based on reduction of ferricytochrome c, a heterologous chemiluminescence-based cell-free system was developed for the detection of low levels of cytosolic activity. This assay has the advantage of heightened sensitivity, and utilises at least 10-fold less cellular material than conventional assays.

Of all chemiluminescence-based assays for NADPH-oxidase function, peroxidase-catalysed oxidation of luminol to chemically excited 3-aminophthalate anion is most sensitive. This reaction is mediated by H_2O_2 formed by virtually instantaneous

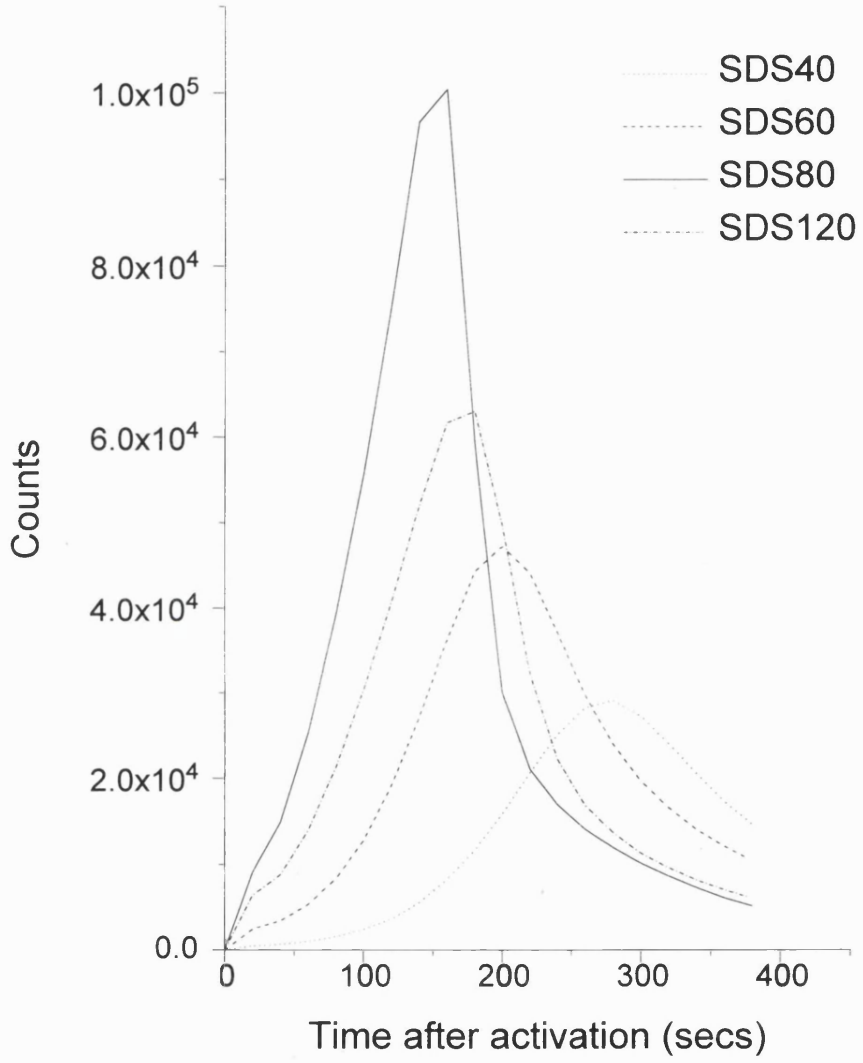
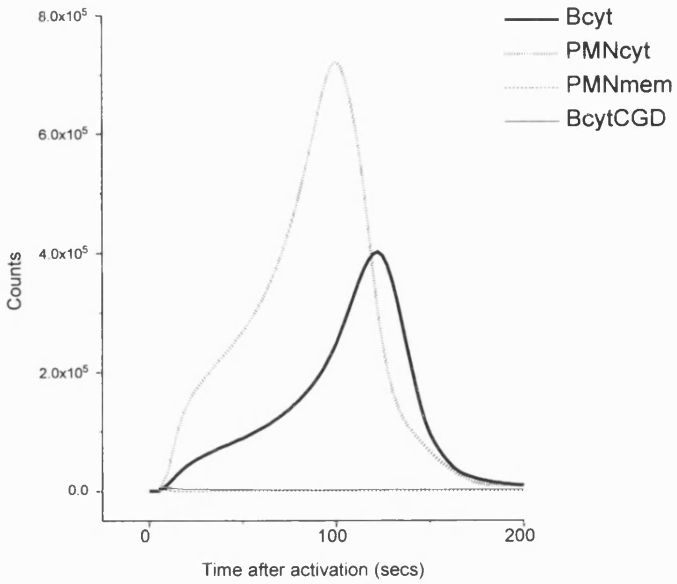


Fig 4.4. Optimisation of chemiluminescence-based detection for products of cell-free NADPH-oxidase activity. Cytosol and membrane fractions purified from human neutrophils were prepared as described in materials and methods. For activation, 2μg of solubilised membrane protein was mixed with 30μg of cytosol in the presence of varying concentrations of SDS (40-120μM), and incubated at room temperature for 2 minutes before addition of assay buffer (65mM sodium phosphate buffer, pH7.0, 1mM EGTA, 1mM $MgCl_2$, 10μM FAD, 10μM luminol, 10U/ml horse radish peroxidase) to a total volume of 100μl, and finally NADPH (0.2mM) to initiate the reaction. Reactions were incubated at 37^{0}C, and all solutions pre-warmed. Maximal activity was achieved at concentrations of SDS between 80 and 120μM, measured on a luminometer.

[A]



[B]

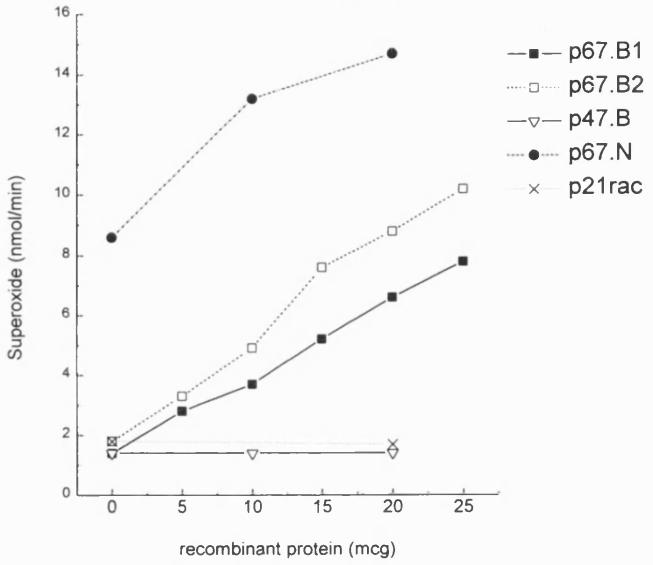


Fig 4.5 Comparison of neutrophil and B cell cytosolic activity in a cell-free assay, and enhancement by exogenous recombinant p67^{phox} protein. [A] Cytosol prepared from normal immortalised B cells (Bcyt), and cells derived from a patient with p47^{phox}-deficient CGD (BcytCGD) were tested for complementation and activation of neutrophil membranes in the chemiluminescence-based cell-free system (see fig 4.4). Consistently, cytosol from neutrophils (PMNcyt) was more active than that derived from B cells. Membranes alone (PMNmem) were inactive. [B] This observation is confirmed in a conventional quantitative assay for production of O_2^- by cytochrome c reduction. For this reaction, 15μg of solubilised membranes were pre-mixed with 200μg cytosolic protein for 2 minutes at room temperature before addition of assay buffer (65mM sodium phosphate buffer, pH7.0, 1mM EGTA, 1mM $MgCl_2$, 10μM FAD, 100μM cytochrome c to a total volume of 1ml, and finally NADPH (0.2mM) to initiate the reaction, which was incubated at 37^{0}C. SDS concentration was optimised for maximal activity (~100μM). Superoxide production was measured for 90s in a double-beam spectrophotometer (Uvikon 860, Kontron). The reference sample was identical, but was supplemented with superoxide dismutase (SOD 50μg/ml). SOD-inhibitable cytochrome c reduction was estimated by differential absorbance at 550nm and 557nm (isobestic points). The reaction was supplemented with affinity purified recombinant p47^{phox}, p67^{phox}, or p21rac1 pre-loaded with GTP (incubated with 100μM GTP in 2mMEDTA for 10mins, followed by addition of 10mM $MgCl_2$). Addition of recombinant p67^{phox} enhanced activity of both neutrophil and B cell cytosol In contrast, recombinant p47^{phox} and p21rac1 produced no effect. The absorption co-efficient (ε) for reduced cytochrome c at 550nm was taken as 21.1mM^{-1}.cm^{-1}.

dismutation of O_2^-. To optimise conditions for a cell-free assay based on this reaction, activity was initially titrated using a cytosolic fraction and a particulate fraction enriched in membranes prepared from human neutrophils. Horseradish peroxidase (HRP) was added to excess, and activation initiated by addition of NADPH. The concentration of SDS in the cell free system is critical for maximal activity to be obtained, and was therefore optimised for each reaction (Fig 4.4). Subsequently, cytosolic extracts prepared from immortalised B cells were mixed with neutrophil membrane fractions. This resulted in optimised maximal activity similar, but consistently less than that using neutrophil cytosol (Fig 4.5A). In contrast, cytosolic fractions derived from patients with $p47^{phox}$-deficient cells were completely inactive. These observations were confirmed in a conventional cell-free assay based on quantitative reduction of ferricytochrome c. Furthermore, supplementation of normal immortalised B cell cytosolic fractions with purified recombinant $p67^{phox}$ restored activity to that of neutrophil cytosol (fig 4.5B). In contrast, addition of recombinant $p47^{phox}$ or GTP pre-loaded (active) p21*rac*1 had no effect. Supplementation of neutrophil cytosolic fractions with recombinant $p67^{phox}$ also enhanced activity, indicating this to be the limiting cytosolic component at least in the cell-free system. In support of these findings, Western blot analysis of B cell cytosolic fractions detected similar levels of $p47^{phox}$ to that of neutrophils, but diminished levels of $p67^{phox}$ (Chetty et al. 1995). Low levels of flavocytochrome in immortalised B cell membranes failed to support significant activity in either assay.

Development of a more sensitive cell-free assay for cytosol-based activity of the NADPH-oxidase is of genuine practical use for exploration of reconstitution methods, and may also provide a clearer insight into the mechanisms of action of this enzyme system in both immortalised B cells and primary phagocytes.

4.3.2 Construction of retroviral vectors and virus producer cell line

Retroviral vectors encoding $p47^{phox}$ were constructed as shown (Fig 4.6). The parent vectors pBabeNeo (shuttle vector) and pBabePuro encode dominantly acting aminoglycoside phosphotransferases which are expressed from an internal SV40 early promoter and which allow growth of mammalian cells in the presence of the

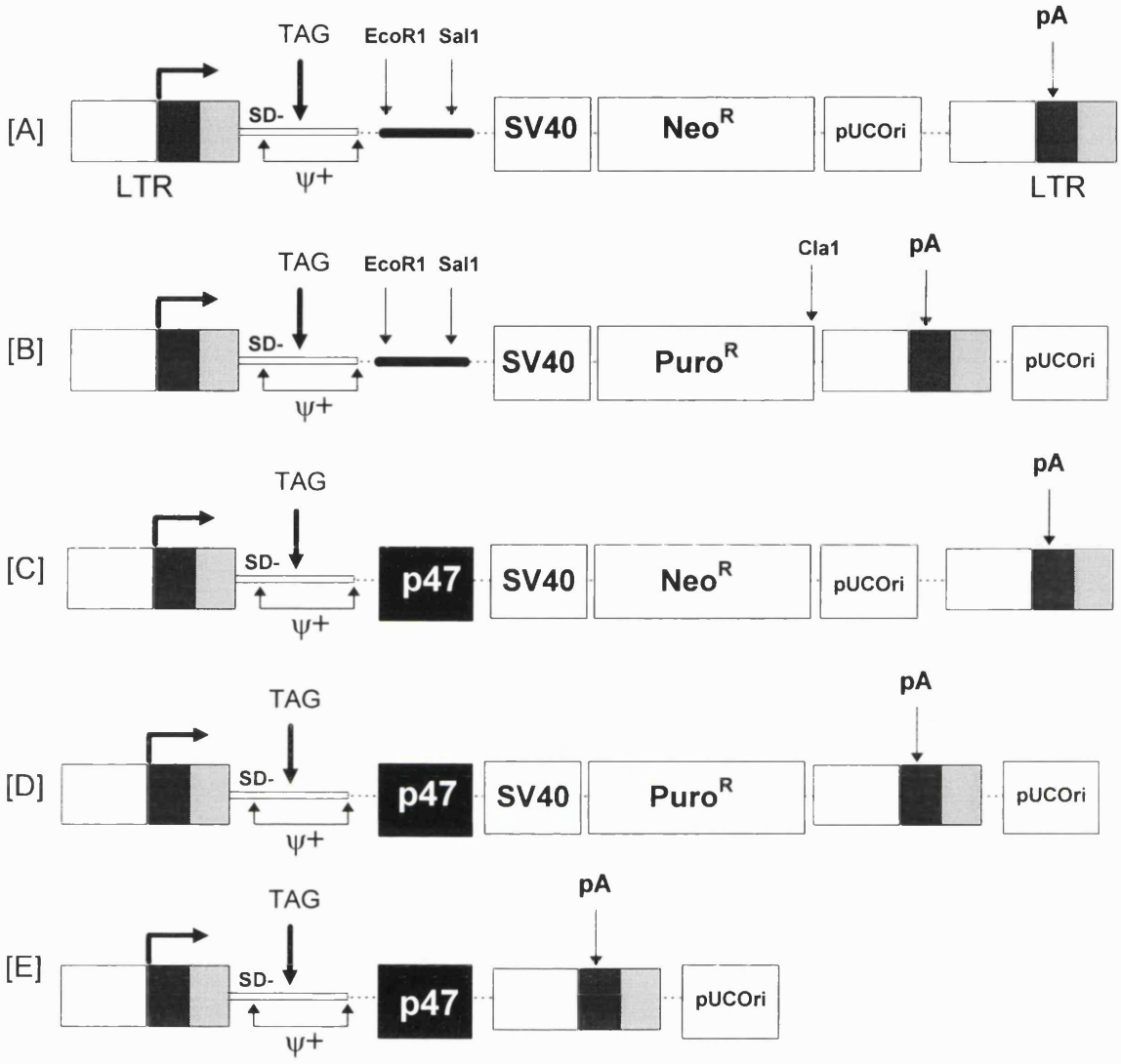


Fig 4.6. MoMLV-based pBabe retroviral vectors.

[A],[B] Parent vectors pBabeNeo and pBabePuro (Morgenstern et al. 1990). Vectors encoding $p47^{phox}$ [C] pBN47, [D] pBP47, [E] pB47. LTR, Long terminal repeat; pA, polyadenylation site; SV40, early promoter; Neo^R/$Puro^R$, drug resistance genes; Black bar, polylinker; pUCOri, bacterial origin of replication; p47, truncated $p47^{phox}$ cDNA; ψ+, retrovirus extended packaging sequence.

[**Construction of retroviral vectors encoding $p47^{phox}$**: A 100bp region of the non-coding 3' sequence including the natural polyadenylation signal, was excised from a full length $p47^{phox}$ cDNA by digestion with Nhe1 and Xba1 (unique cloning vector polylinker site). Following recircularisation, the truncated cDNA was isolated by digestion with EcoR1 and Sal1, and cloned into corresponding sites in the polylinker of pBabeNeo and pBabePuro. To construct pB47, the puromycin resistance expression cassette was excised from pBP47 by digestion with Sal1 and Cla1.]

neomycin analogue G418, and puromycin, respectively (Morgenstern and Land, 1990). pBabe vectors, based on MoMLV, incorporate a mutagenised *gag* cassette lacking the natural initiator codon (ATG mutagenised to TAG) and including the extended packaging sequence (ψ+), and a splice donor point mutation (SD-) inserted to avoid splicing artefacts. The multiple cloning site permits expression of inserted genes from the retroviral LTR.

pBabeNeo and pBabeNeo47 (pBN47) vectors were introduced into the PA317 packaging cell line by calcium phosphate co-precipitation, and colonies resistant to G418 (1mg/ml) isolated by ring cloning. Of twenty clones from each, one producing the highest titre on NIH 3T3 cells (~10^5 cfu/ml) was expanded and used for investigation (PANeo.1 and PA47Neo.1). pBabe47 (pB47) was transfected into ΩE and GP+AM12 packaging cell lines together with pSV2Neo (ΩE was used in preference to GP+86 because this line is specifically designed for pBabe, and because laboratory stocks of GP+86 failed to produce virus when transfected with a vector genome. This may reflect instability of packaging constructs arising from the method of insertion). Single clones of G418 resistant GP+AM12 cells were expanded, and screened for highest titre by slot-blot hybridisation. Best producers were titred by Southern blot analysis. One clone producing the equivalent of ~10^5 cfu/ml (AM47.1) was expanded and sequentially infected with supernatant harvested from the transfected polyclonal ΩE producer cell line. Final titre was equivalent to ~5.10^5 cfu/ml (AM47.2).

4.3.3 Infection of immortalised B cells

10^6 viable immortalised B cells derived from patients with $p47^{phox}$-deficient CGD, were co-cultured with PANeo.1 and PA47Neo.1, respectively, in the presence of polybrene (8μg/ml). After 48hrs, non-adherent cells were removed and grown in fresh medium supplemented with G418 (2mg/ml). Unmanipulated cells were previously shown to survive less than two weeks under these conditions. After 6 weeks growth, sufficient numbers of G418-resistant cells were available for testing.

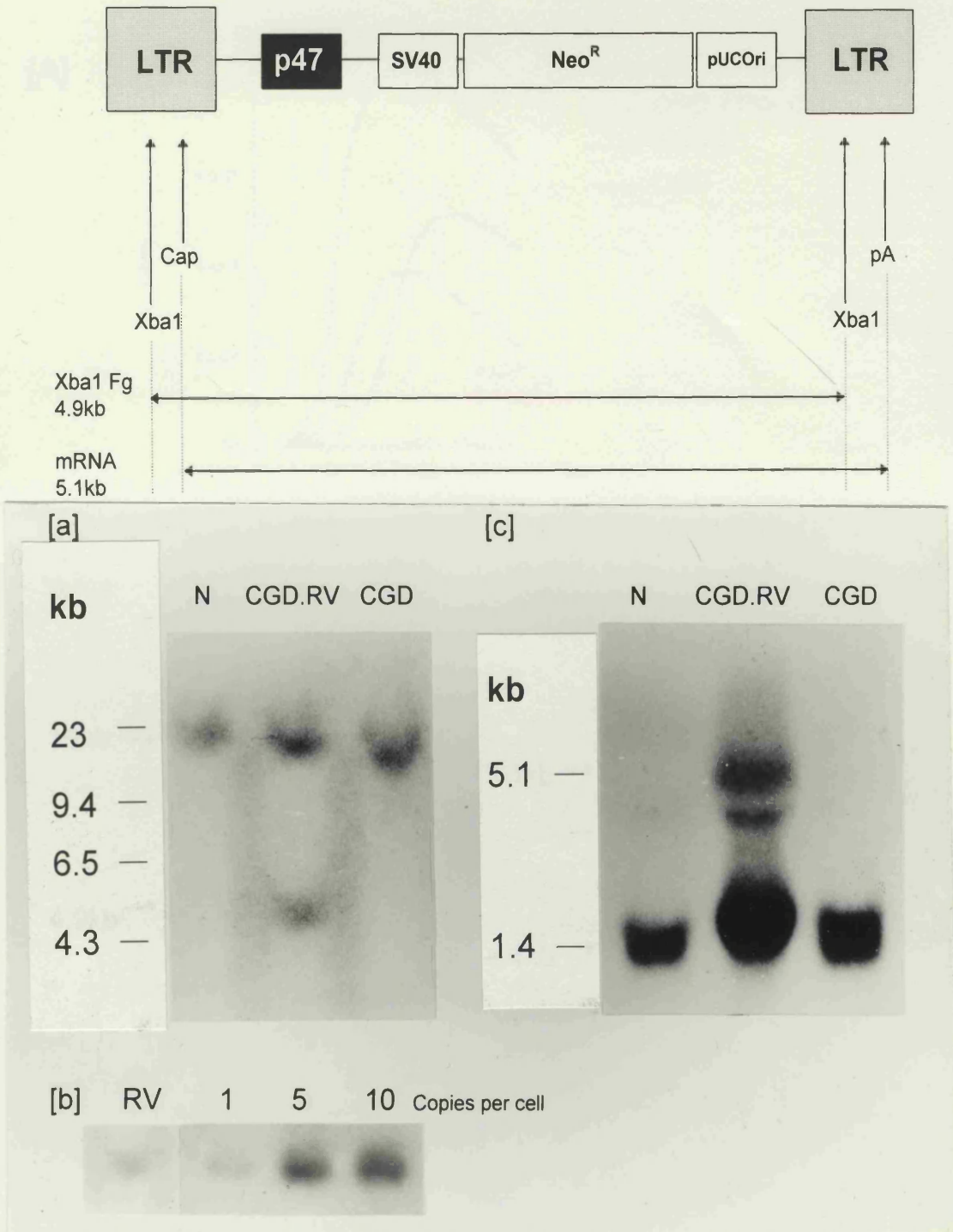


Fig 4.7. Detection of proviral genome and corresponding mRNA species. [a] Southern Blot showing Xba1 digested genomic DNA (10µg) prepared from normal cells (N), $p47^{phox}$-deficient cells (CGD), and the same cells transduced by pBN47 retrovirus (CGD.RV), and cultured in the presence of G418 (2mg/ml) for six weeks. The fragment released by digestion in each LTR of the provirus is 4.9kb, in contrast to the19kb natural genomic fragment. [b] Estimation of copy number: The Xba1 digested genomic fragment from the transduced cells (RV), and copy number equivalents from Xba1 digested plasmid DNA, 1,5, and 10 copies per cell, were electrophoresed and blotted together. From this, and from [a] using the natural gene as an internal control (2 copies), the proviral copy number can be estimated to be close to one. [c] Northern blot of total RNA extracted from same cells. A 1.4kb species is present in all cells and represents the natural $p47^{phox}$ mRNA. In contrast, the 5.1kb provirus-specific message is found only in transduced cells. All blots were probed with [^{32}P]dCTP- labelled $p47^{phox}$ cDNA, and washed to a final stringency of 2xSSC, 0.1%SDS at 65^{0}C.

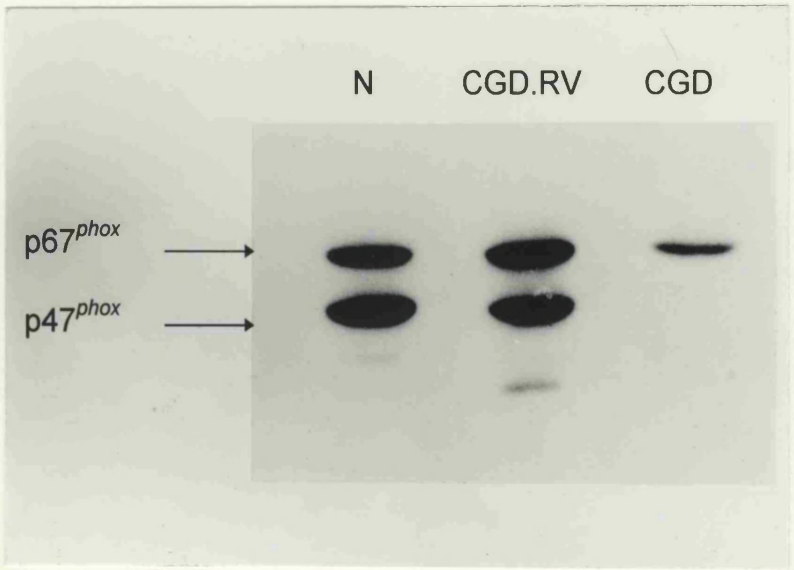


Fig 4.8.Western blot analysis of transduced cells. Cells were disrupted by sonication, and crude extract separated on a 12.5% polyacrylamide gel. After transfer to nitro-cellulose, blots were incubated with specific polyclonal antisera to $p47^{phox}$ and $p67^{phox}$. Bands were visualised by reaction with [^{125}I]-labelled protein A. Extracts were prepared from normal cells (N), transduced CGD cells (CGD.RV), and untransduced cells from the same patient (CGD). In order to normalise for loading, the relative amounts of $p67^{phox}$ protein in each lane was estimated densitometry. From these measurements, it can be estimated that approximately 50% of normal levels of $p47^{phox}$ protein was expressed in transduced cells.

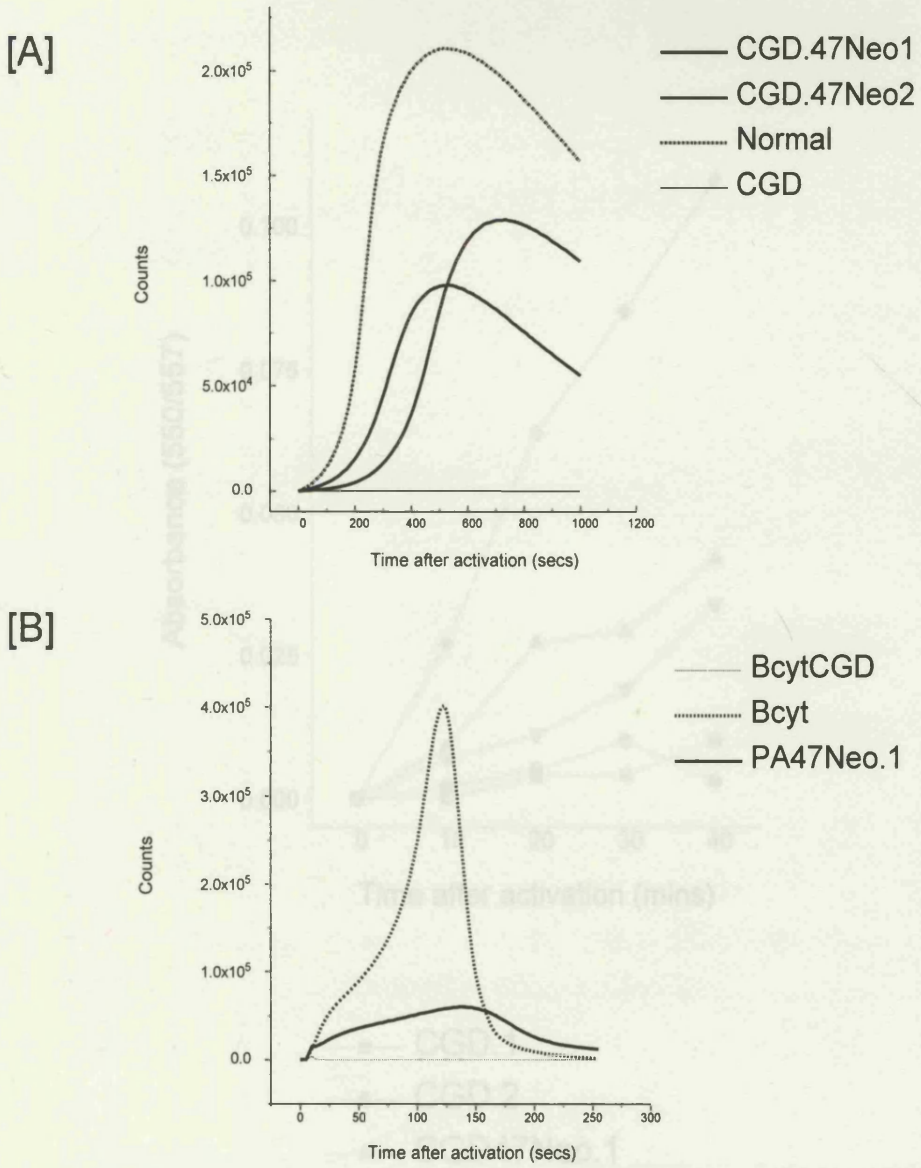


Fig 4.9. Chemiluminescent assay for activity in transduced cells. [A] To assay for activity in whole cells, 5.10^6 cells were washed twice with PBS, and resuspended to a total volume of 1ml in HBSS with calcium and magnesium (0.5 mM and 1mM, respectively), 10μM luminol and 10U/ml horse radish peroxidase. Activation was initiated by the addition of PMA (1μg/ml) at 37^0C, and superoxide production measured in a luminometer as before. CGD cells from two patients, co-cultivated with the producer cell line PA47Neo.1 in the presence of polybrene (8μg/ml) and grown for 6 weeks in selection (CGD.47Neo1 and CGD.47Neo2), both showed considerable restoration of function compared with normal cells (normal), and untransduced cells from either patient (CGD). Cells derived from a patient with X-linked disease, transduced by the same vector, were inactive (not shown). Similarly, cells transduced by the parent vector encoding resistance to G418 were also inactive (not shown). [B] Chemiluminescence-based cell-free assay (see fig 4.5A) for activity in transduced CGD B cell cytosol (PA47Neo.1), compared to normal B cell cytosol (Bcyt), untransduced CGD cytosol (BcytCGD). Activity from neutrophil membranes alone was indistinguishable from background (not shown).

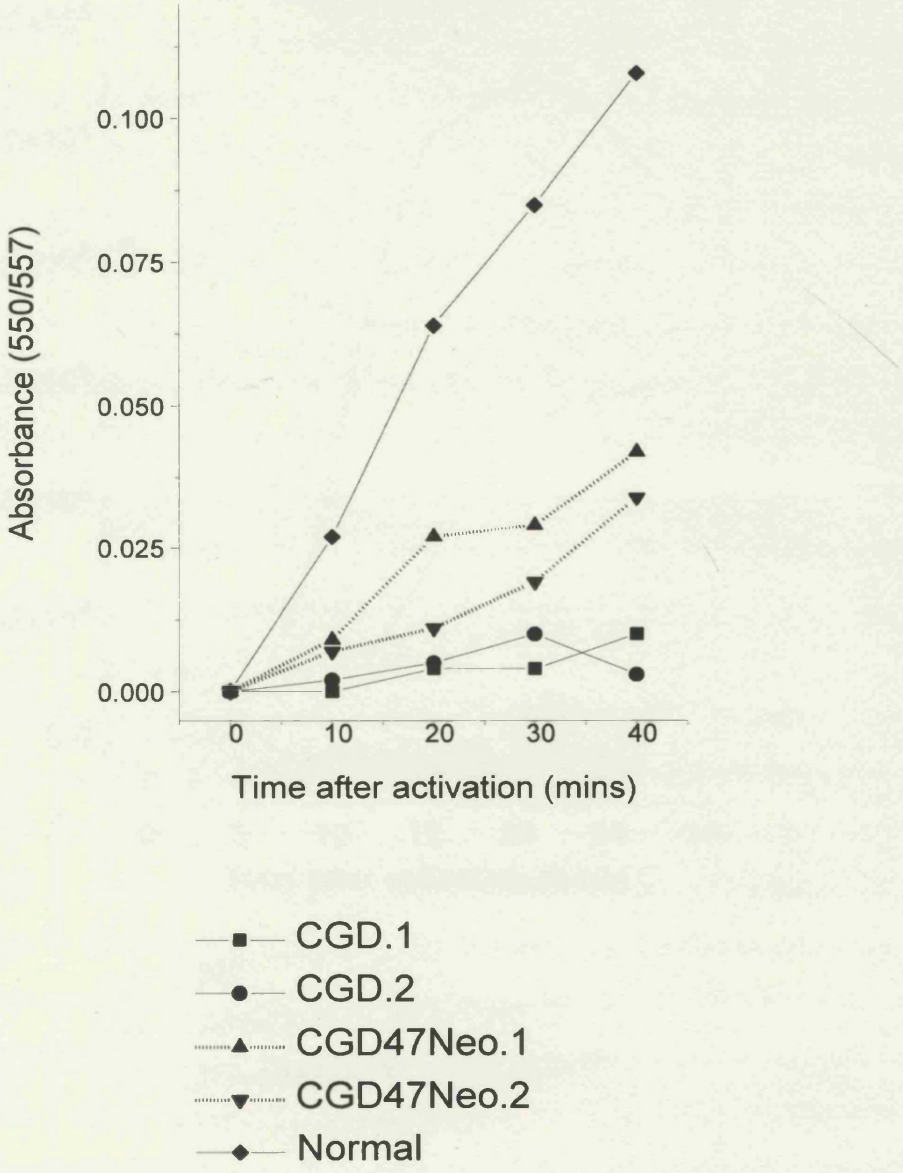


Fig 4.10. Quantitative assay for production of superoxide by reduction of ferricytochrome c. For this assay, 5.10^6 washed cells were resuspended in HBSS with calcium and magnesium at a final concentration of 2.10^6 cells per ml, in the presence of 100μM ferricytochrome c and 150μM NADPH. Reaction was initiated by the addition of PMA (1μg/ml), and incubated at 37^0C with gentle mixing. 0.5ml aliquots were removed from the reaction at 10 minute intervals, and quenched by addition of 1ml of ice-cold PBS, and centrifugation at 4000rpm in a microfuge at 4^0C. A parallel reaction was incubated in the presence of SOD (50μg/ml), and SOD-inhibitable reduction of cytochrome c measured by differential absorbance at 550nm and 557nm (isobestic points) in a dual beam spectrophotometer (Uvikon 860, Kontron). Based on an extinction co-efficient for cytochrome c at 550nm of $21.1mM^{-1}.cm^{-1}$, two transduced CGD cell lines (CGD47Neo.1 and CGD47Neo.2) produced approximately 30% (0.38nmol/10^7 cells per minute), and 36% (0.47nmol/10^7 cells per minute) of the activity obtained from the reference cell line (normal). In contrast, untransduced cells produced little activity (CGD.1 and CGD.2).

The presence of the recombinant genome is indicated on a Southern blot hybridised to a [^{32}P]-dCTP-labelled p47^{phox} cDNA probe (Fig 4.7A). By comparison to the intensity of signal generated from the endogenous gene (2 copies per cell), and from dilutions of purified fragment, the number of copies of the recombinant genome in the majority of cells can be estimated as close to one (Fig 4.7B). On a Northern blot, the same cDNA probe identified a 5.1kb mRNA species specific to pBN47 transduced cells, which is the predicted size of a full length proviral transcript, in addition to endogenous message (1.4kb) (Fig 4.7C). In confirmation of successful transduction and gene expression, a highly specific polyclonal antiserum raised against the C-terminal 13 amino acids of p47^{phox} identified a 47kDa protein only in those patient-derived cells which had been co-cultivated with PA47Neo.1 (Fig 4.8). The level of protein expression in these cells compared to that of normal cells was estimated to be 50% by densitometry, using p67^{phox} as an internal control for loading.

4.3.4 Restoration of NADPH-oxidase function to immortalised B cells

NADPH-oxidase activity in transduced immortalised B cells derived from two patients with p47^{phox}-deficient CGD is shown in (Fig 4.9A). For both patients, co-cultivation with PA47Neo.1 restored significant activity, measured by chemiluminescence, to PMA-stimulated cells in comparison with similar cells derived from a normal individual. Unmanipulated cells, or those exposed to PANeo.1 were inactive. Confirmation that activity was restored specifically to the cytosolic fraction in a cell-free assay was demonstrated by complementation with a particulate fraction of neutrophil extracts enriched for membranes (Fig 4.9B). By ferricytochrome c reduction, cells transduced by the retroviral vector encoding p47^{phox} produced approximately between 30% and 36% of the activity produced by a similar number of reference cells, and fits well with the level of protein expression shown earlier (Fig 4.10). In contrast, untransduced cells from these patients produced undetectable levels of O_2-. Similarly, cells transduced by the basic retroviral vector pBabeNeo, and grown in G418 selection (2mg/ml) for six weeks showed no restoration of function measured by all assays (not shown). Subsequent studies investigated transduction of p47^{phox}-deficient immortalised B cells using the AM47.2 producer cell line. This proved to be considerably more efficient than PA47Neo1, and restored activity of deficient B cells to

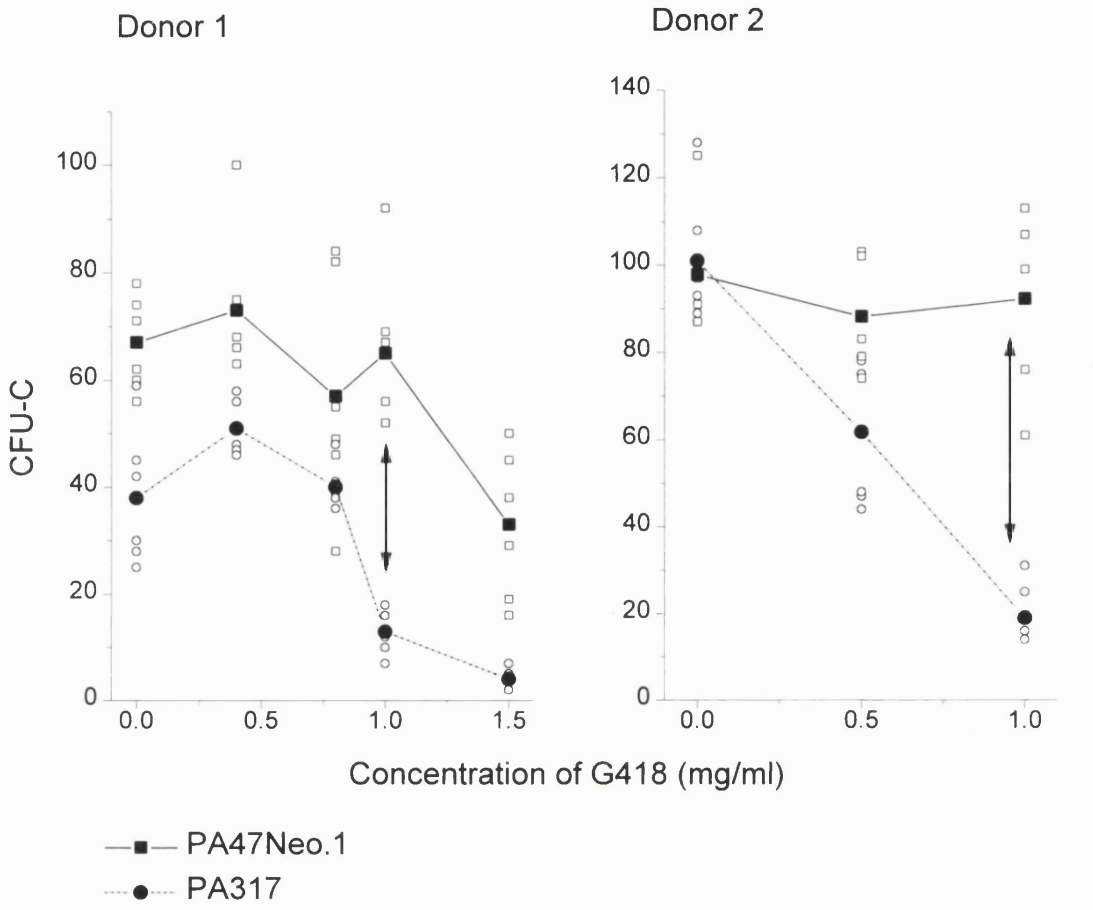


Fig 4.12. Transduction of low density mononuclear cells derived from bone marrow harvested from two normal adult human donors (donor1 and donor2). Cells were prepared from 10-20 mls of whole bone marrow as described in materials and methods, and were co-cultured (Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS and 10% HS) for 48hrs with confluent irradiated (1500cGy) producer cell lines, PA47Neo.1 or the PA317 parent cell line, at a concentration of 1-2.10^6cells per ml, in the presence of polybrene (2μg/ml), IL-3 (20ng/ml), GM-CSF (10ng/ml) and SCF (12ng/ml, conditioned medium). After this period, cells were washed from the producer line, and plated in 1.3% semi-solid methylcellulose for clonogenic assay, in the presence of G418 (0-1.5mg/ml). For donor1, cells were incubated in fresh medium containing G418 (1mg/ml) overnight before plating. Clonogenic progenitor-derived colonies were scored by light microscopy after 14 days (CFU-C). Open symbols represent the actual number of colonies in 5 replica plates for each concentration of G418. Solid symbols represent the mean number of colonies. The bold arrow indicates the concentration of G418 at which the greatest difference could be observed between transduced (PA47Neo.1), and sham-transduced (PA317) populations, and is probably the best indication of transduction efficiency.

comparable levels in the absence of selection. More importantly, functional reconstitution was stable in tissue culture for at least 4 months post transduction (not shown).

4.3.5 Transduction of human bone marrow

Low density mononuclear cells derived from normal individuals were isolated as described in materials and methods, and co-cultured with irradiated producer cells PA47Neo.1, or the parent cell line PA317 for 48 hours in the presence of IL-3 (20ng/ml), GM-CSF (10ng/ml) and SCF-conditioned medium (produced from an engineered CHO cell line, and used at 12ng/ml final concentration of SCF). Non adherent cells including the clonogenic progenitors were plated in semi-solid methylcellulose in the presence of G418 at varying concentration (0-1.5mg/ml). After 10-14 days, plates were examined and scored by light microscopy for the formation of colonies (CFU-C). For the two experiments shown, cells exposed to PA47Neo.1 gave rise to significantly greater numbers of resistant colonies than those co-cultured with the parent PA317 cells, when grown in the presence of G418 (Fig 4.12). The concentration of G418 at which the greatest difference is observed is marked by an arrow, and probably reflects successful gene transfer. However, the concentration of G418 required to prevent growth of untransduced colonies, yet permit growth of those successfully transduced, varied considerably between patient samples, even though co-culture and growth conditions were standardised as much as possible. Consistency was therefore difficult to achieve, and efficiency of transduction of clonogenic progenitors assayed in this way ranged from 5 to 90% over several experiments. Subsequently, for experiments using the AM47.2 producer cell line, gene transfer to clonogenic progenitors was assayed by PCR using primers specific to the proviral genome. By this method, efficiency of transduction under similar culture conditions averaged between 30-40% (J.Povey, personal communication).

4.4 DISCUSSION

Primary phagocytic cells are not good candidates for retrovirus-mediated complementation of the CGD phenotype because of transcriptional quiescence in the

case of neutrophils, and the reluctance of both these cells and monocytes to enter cell cycle. Fortunately, EBV-immortalised B cell lines provide an alternative parallel model system. These studies demonstrate correction of the biochemical phenotype typical of CGD in intact cells, and endorse a gene transfer approach for curative therapy of this disorder (Thrasher et al. 1992). Attempts to extend these studies to primary monocytes derived from patients with $p47^{phox}$-deficient CGD were unsuccessful primarily because of growth quiescence of these cells even in the presence of stimulatory cytokines such as IFN-γ and M-CSF (not shown). Subsequently, immortalised B cell lines deficient in other components of the NADPH-oxidase have been successfully reconstituted using both retrovirus-based and episomal plasmid systems, although the latter are unlikely to be applicable to a therapeutic strategy (Chanock et al. 1992; Maly et al. 1993; Porter et al 1993; Volpp and Lin, 1993; Porter et al. 1994).

The levels of reconstitution achieved in lymphoblastoid cells compare favourably with activity of similar cells derived from normal individuals, and reflect both the activity of the MoMLV LTR and stability of message. Increased efficacy of a producer cell line in which the retroviral vector did not encode a dominant selectable marker or bacterial origin of replication (AM47.2) probably reflects greater activity of the LTR in the absence of a competing promoter, and enhanced stability of a shorter message, in addition to benefits derived from higher viral titre. Constitutive differences between the parental packaging cell lines (PA317 and GP+AM12), particularly with regard to cytokine secretion may be influential, but is less likely to be so for immortalised cell lines which are selected for continued growth, than for primary cells (Xu et al. 1994). The activity of the MoMLV in myeloid cells is clearly of interest, and in agreement with other studies, levels of $p47^{phox}$ transcript expressed from both from the natural gene and from an integrated retrovirus are induced co-incident with differentiation of the myeloid cell line HL60 (not shown) Reisman and Rotter, 1989). Lineage-dependent expression for this virus was not tested, but on the basis of other studies, would not be expected to show a restricted pattern.

These preliminary experiments also demonstrated successful transduction of clonogenic progenitor cells derived from normal human bone marrow, with a vector encoding

$p47^{phox}$, at an average frequency of between 30-40%. However, for reasons discussed previously, expression of drug resistance is unlikely to reflect expression of other genes inserted in the same vector and expressed from a competing promoter, and can only therefore be useful as a determinant of successful gene transfer. Even measurement of this parameter is complicated by the assay used, namely the ability of cells to grow in the presence of the neomycin analogue, G418. Variability of growth-inhibiting activity between different patient samples, and under different conditions of cell culture has been noted by other groups, suggesting that such data should be interpreted at least with caution, and that alternative methods for assay of transduction efficiency, such as that based on PCR are preferable. Furthermore, expression of neomycin phosphotransferase may interfere with the growth characteristics of selected clonogenic cells (unpublished).

The implications and significance of these results for gene therapy of CGD will be discussed later.

4.5 SUMMARY: RETROVIRUS-MEDIATED GENE TRANSFER FOR CGD

- *NADPH-oxidase function can be restored to immortalised B cells derived from patients with autosomal recessive CGD by retrovirus-mediated gene transfer. Levels of activity in these cells compare favourably with those from normal individuals.*

- *The same recombinant retroviruses transduce haematopoietic progenitor cells derived from human bone marrow.*

- *Efficiency of gene transfer, and gene expression, are enhanced by exclusion of G418-resistance markers from the retroviral vector.*

CHAPTER 5:

ADENO-ASSOCIATED VIRUS GENE TRANSFER

5.1 INTRODUCTION

The feasibility of using gene transfer to correct the biochemical defect characteristic of CGD has been successfully demonstrated by retrovirus-mediated reconstitution of NADPH-oxidase activity in immortalised B cells derived from patients (Thrasher et al. 1992), and more recently by correction of mature phagocytic cells derived *in vitro* from transduced progenitor populations (Sekhsaria et al. 1993; Li et al. 1994). However, successful transduction of relatively quiescent cells, such as pluripotent haematopoietic stem cells (PHSC), is limited by the dependence on mitosis of the current generation of murine retrovirus-based vector systems. Their potential to mediate lifelong correction of disease phenotype is therefore restricted. An alternative vector system which is less dependent on cell cycle, and which may result in stable transduction, is based on the non-pathogenic human virus, adeno-associated virus (AAV) (Muzyczka, 1992; Kotin, 1994).

5.2 ADENO-ASSOCIATED VIRUS VECTOR SYSTEMS

5.2.1 Adeno-associated virus life-cycle

AAV is a member of a family of small non-enveloped icosahedral viruses, the *Parvoviridae* (Carter, 1990a). In contrast to the autonomous viruses, such as human parvovirus B19, the *Dependoviruses*, of which AAV is the only described member, require co-infection with helper viruses, usually adenovirus (Ad) or herpes simplex virus, for efficient replication and propagation during the lytic phase of the viral life cycle. In the absence of helper function, AAV establishes a latent infection by integration into the cellular genome at a preferential chromosomal site on chromosome 19q13.3-qter (Kotin, 1990, 1991, 1992; Samulski, 1991). Super-infection of latently

infected cells with helper virus results in rescue and amplification of the AAV genome, propagation of infectious particles, and continuation of the virus life-cycle.

AAV was originally isolated as a contaminant of adenovirus preparations, but in isolation has not been causally associated with human disease, even though 85% of the adult population are sero-positive (Blacklow et al. 1968). AAV may even be inhibitory for adenoviral replication (Casto et al. 1967; Carter et al. 1979) and may protect against virus-mediated cell transformation (Hermonat et al. 1989; Heilbronn et al. 1990; Kleinschmidt et al. 1995). Host range for productive infection is primarily determined by the helper virus, but the range of cells in which latent infection can be established is much wider, and extends to other species. The cellular receptor or receptors for AAV are undefined, but for the related autonomous parvovirus B19 is the erythrocyte glycolipid P antigen (Brown et al. 1993).

5.2.2 AAV structure

At least ten serotypes of AAV have been isolated, the best characterised of which is AAV-2. Mature AAV particles are between 20 and 24nm in diameter, equilibrate in a density gradient at 1.41g/cm^3, and are relatively resistant to heat (56^{0}C for one hour), low pH, and disruption by protease or detergent action (Arella et al. 1990). The viral capsid is composed of three proteins, VP1, VP2, and VP3, of which VP3 is the most abundant (90%).

The AAV-2 genome consists of a single-stranded (ss) linear DNA molecule of 4680 nucleotides, which is packaged as a (+) or (–) strand with equal efficiency (Fig 5.1). At opposite ends of the genome are two 145 base inverted terminal repeat (TR) sequences, of which the first 125 nucleotides can form a T-shaped hairpin structure by internal base-pairing (Carter et al. 1990b). Between the TR sequences are two major open reading frames (ORF) encoding the three structural capsid proteins VP1-3 (Cap), and four proteins necessary for replication, encapsidation, site-preferential integration, and rescue of the AAV genome from latency, Rep78, Rep68, Rep52, and Rep40 (Rep). Messenger RNA is initiated from three promoters described by their map positions. The p5 and p19 promoters initiate expression of the larger Rep proteins, Rep78 and Rep68,

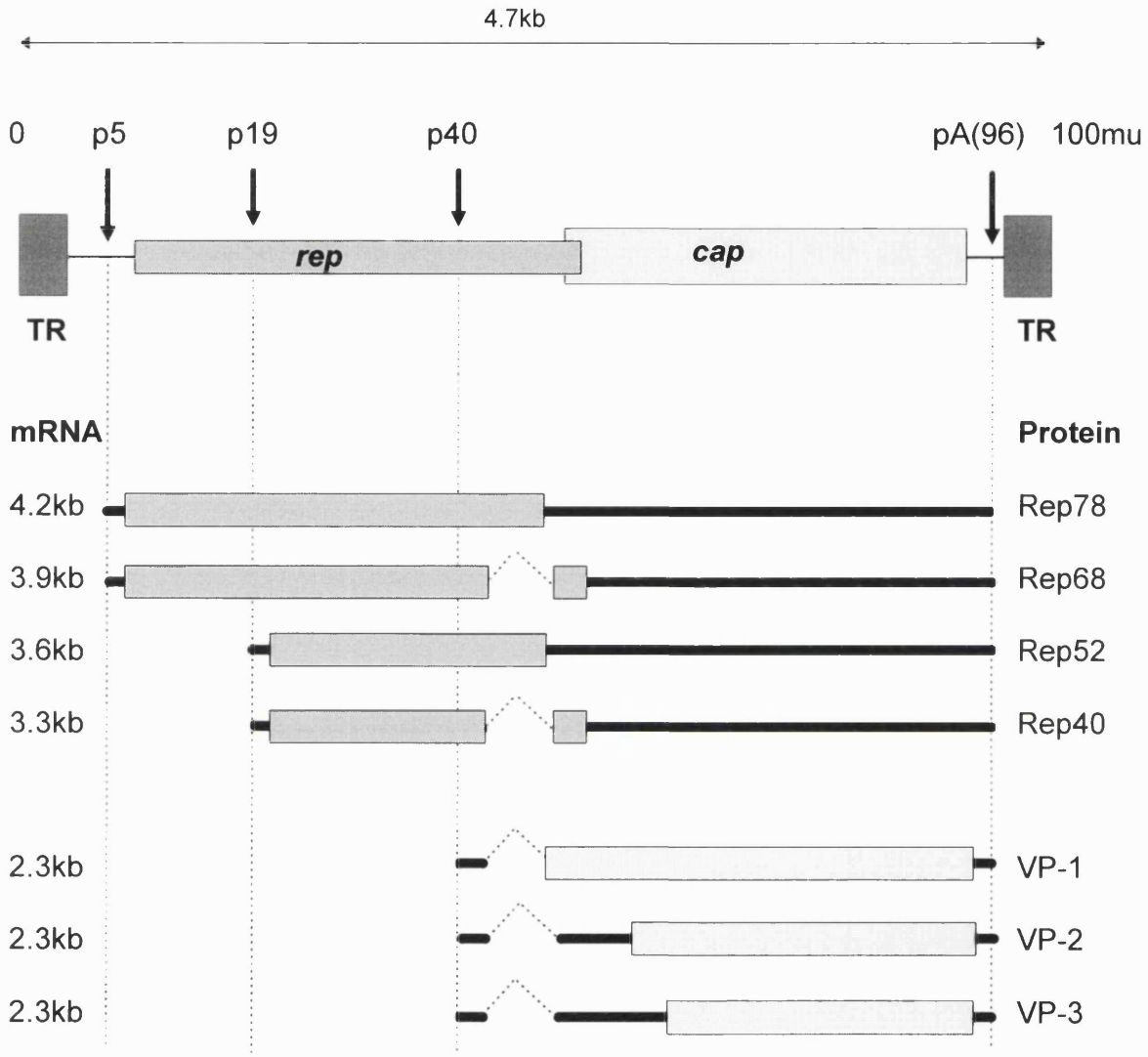


Fig 5.1. Genetic map of full length AAV genome (4680nt). The 145 nucleotide terminal repeat sequences are represented by TR, and the the three major promoters by their map position p5, p19, and p40 (mu, map units). The two major open reading frames (ORF) encoding *rep* and *cap* are indicated, along with the major transcripts derived from the AAV promoters which are shown by a heavy line. Translated regions are indicated by shaded boxes. The common intron (nucleotides 1907-2227) is represented by the dotted line. Rep translation products are denoted by molecular weight in kDa. Viral capsid protein-1 (VP-1) utilises an initiation codon within the common intron, accessed by use of an alternative splice acceptor at nucleotide 2201, which permits expression of the entire right open reading frame. VP-2 is synthesized from a longer VP-3 ORF by use of an ACG initiation codon at nucleotide 2615. The polyadenylation signal at map position 96 is common to all transcripts.

and the smaller proteins, Rep52 and Rep40, respectively, of which the latter are identical to the C-terminal sequences of the larger translation products. The p40 promoter directs expression of the three capsid proteins. All AAV transcripts share a common intron between nucleotides 1907 and 2227, which is alternatively spliced to create each pair of *rep* mRNA species, although one p40 mRNA species utilises an alternative splice-acceptor site at nucleotide 2201.

5.2.3 AAV DNA replication

Replication of the AAV DNA genome is dependent on host cellular enzymes. The two regions of the AAV genome necessary for DNA replication are the origin of replication in the TR, and the *rep* gene. TR sequences are required in *cis* for AAV DNA replication, encapsidation, at least for random integration, and for rescue from chromosomal DNA, or from plasmid sequences. The larger Rep proteins, Rep78 and Rep68, are the only obligatory AAV-specific gene products. Mutations in Rep52 and Rep40 do not affect accumulation of replicative-form (RF) DNA, but may prevent accumulation of ss progeny DNA (Chejanovsky and Carter, 1989). A self-priming strand displacement model for AAV replication is based on the mechanism proposed for the replication of eukaryotic chromosome ends (Cavalier-Smith, 1974). The major replicative forms (RF) are a linear duplex molecule with open ends, and one in which the ends are covalently joined (Fig 5.2). Dimer concatamers arise by initiation of replication on unresolved monomers. Cap mutants fail to accumulate ss progeny DNA, despite synthesising normal amounts of RF species, indicating a requirement for preformed capsids to mediate encapsidation, but not strand displacement (Hermonat et al. 1984; Tratschin et al. 1984).

Rep proteins possess several properties which allow the replicative process, and resolution of genomic ends to occur. These include binding to the TR (Im and Muzyczka, 1989), site and strand-specific endonuclease activity which nicks the terminal resolution site (*trs*) (Im and Muzyczka, 1990), and ATP-dependent DNA-helicase activity (Im and Muzyczka, 1992). Rep protein bound to the TR catalyses a single-strand nick at the *trs*, and becomes covalently linked to the newly generated 5' end (Snyder et al. 1990). The free 3' end then primes DNA synthesis complementary

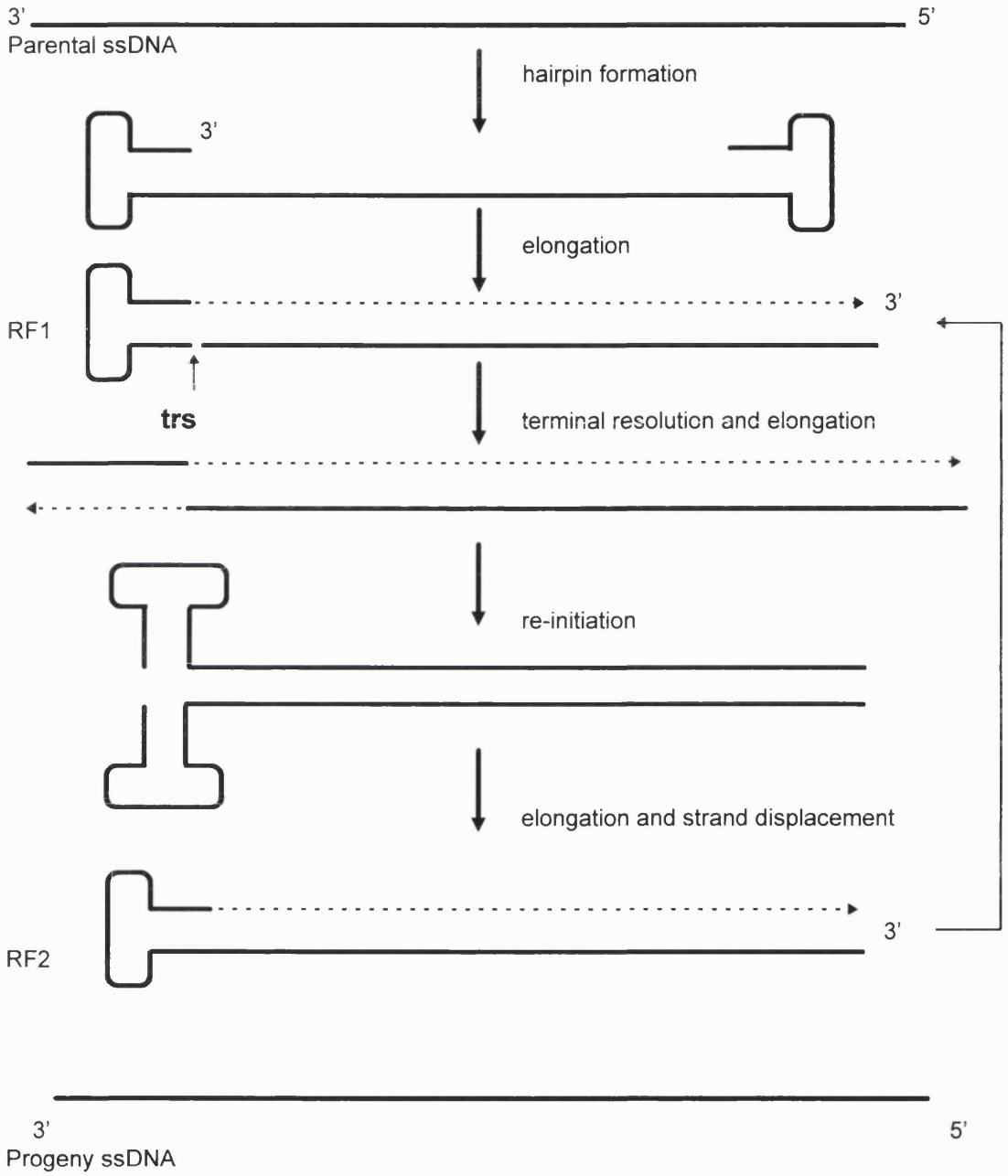


Fig 5.2. AAV DNA replication cycle. The terminal palindrome acts as a primer for synthesis of a duplex linear molecule covalently closed at one end, the replicative form (RF1). Upon binding of the hair-pinned terminal repeat (TR), the Rep protein nicks the bottom parental strand at the terminal resolution site (trs) opposite the original 3' primer position, and becomes covalently linked to the 5' end of the cut strand. The 3' OH primer generated by the nick is used to repair the terminal sequence in a process called terminal resolution. Re-initiation and displacement synthesis releases a single-stranded progeny molecule (ss), and a new replicative form (RF2) from which a new round of replication can initiate. Accumulation of ss progeny DNA is probably dependent on the production of AAV capsids.

to the A, B and C palindromes, a process which inverts the orientation of the B and C palindromes relative to the *trs* (Fig 5.3). A recognition element in the A stem (including an imperfect $[GCTC]_4$ repeat), and a functional *trs* are sufficient to direct the site and strand-specific nick even in the absence of secondary hairpin structure produced by the B and C palindromes (McCarty et al. 1994a). *Trs* endonuclease activity is less efficient in this linear configuration, although the stability of binding to the A-stem is probably unchanged, indicating that the two processes are distinct (Chiorini et al. 1994; McCarty et al. 1994b).

5.2.4 AAV gene regulation

AAV gene expression is regulated by *cis*-acting elements in the AAV genome, and *trans*-acting factors expressed from *rep* genes, helper virus, and the host cellular genome. Latently infected cells express little AAV message or protein, partly due to self-repression of *rep* gene transcription mediated by Rep78 and Rep68 (Beaton et al. 1989). Infection by helper virus results in a cascade of events optimising expression of factors necessary for propagation of AAV. Under these circumstances, Rep proteins are capable of transactivating all AAV promoters in the absence of the TR, but for optimal activity require the presence of the p5 and p19 promoter sequences in *cis*. An intact TR can substitute for these two promoters *in cis*, suggesting that Rep mediates complex formation between the three sites (McCarty et al. 1991; McCarty et al.1994b). Evidence for binding to the p5 promoter through a repeating GAGC motif (nucleotides 260-284 between the TATA-box and the proximal YY1 binding site) similar to that in the A-stem suggests that Rep, YY1 and TATA-binding protein interact to determine repression or activation of transcription depending on the latency of infection (fig 5.4). There is recent evidence that Rep binds to the p19 promoter, and possibly the p40 promoter (McCarty et al. 1994b).

Some elements of the AAV promoters have been functionally defined, most significant of which are binding sites for the ubiquitous and multifunctional cellular transcription factor YY1 (Fig 5.4). YY1 is a DNA-binding protein containing a zinc finger domain, related to the Drosophila Kruppel family of transcriptional regulators. YY1 has been shown to be a transcriptional activator or repressor depending on cellular context,

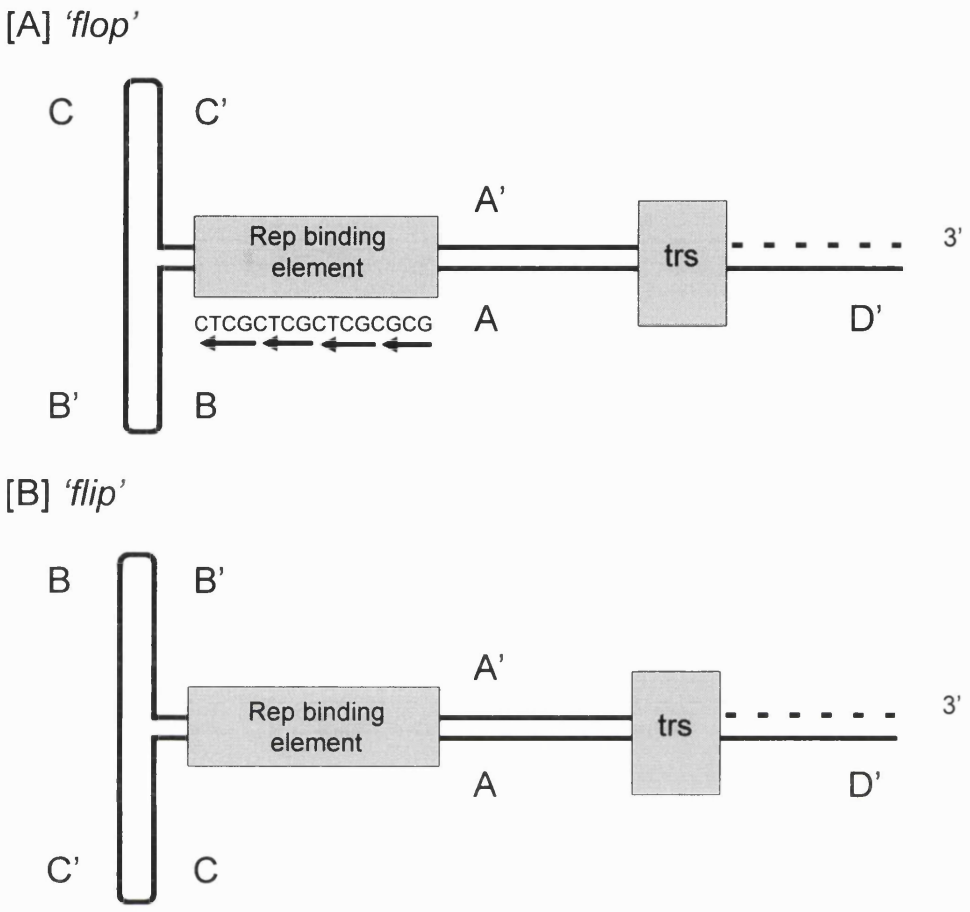


Fig 5.3. [A] and [B] Structure of the 145 nucleotide terminal repeat (TR). The TR as shown above is composed of three palindromic sequences, A,B, and C, which fold into a hair-pinned T-shaped structure in replicating DNA. The D region extends from nucleotides 126 to 145. The stem is formed by the A region and is a 40 nucleotide duplex of which two thirds of base pairs are G-C. The T region consists of two stems of eight or nine base pairs which with one exception are 100% G-C. As a result of terminal resolution, regions B and C invert relative to the remainder of the sequence, and may therefore appear in two orientations 'flip' (B) and 'flop' (A). Both ends in any single molecule have an equal probability of being in either configuration. The Rep binding element is a 25bp region within the A stem containing 4 imperfect [GCTC] repeats which is necessary for interaction with the larger Rep proteins, and 'trs' the minimal sequence necessary for terminal resolution [5' AGTTGG 3'].

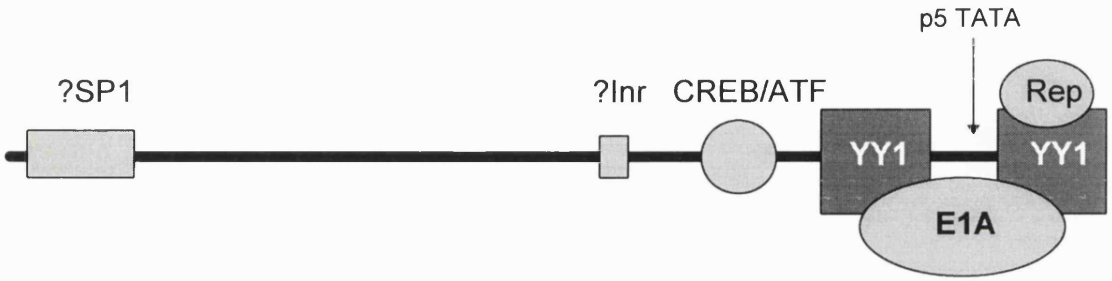


Fig 5.4. Transcription factor binding sites within the first 300bp of a double stranded linear AAV genome. The TATA-box of the p5 promoter lies at nucleotide 255. Nucleotides 160 to 180 constitute a binding site for CREB/ATF family of transcription factors. Two regions surrounding the p5 promoter are binding sites for YY1, the first between nucleotides 215 and 235, and the second overlying the transcription initiation site. This latter region has also been shown to bind Rep protein. Adenovirus E1A protein relieves repression imposed by YY1, which then transactivates the p5 promoter. Putative SP1 binding sites are indicated, together with an Inr consensus within the D region of the terminal repeat, which may account for weak promoter activity of isolated TRs.

and to function as an initiator for a number of genes. An effect on promoter topology may be an important determinant of interaction with other regulatory proteins (Natesan et al. 1993). Two binding sites for YY1 have been isolated from the p5 promoter, which in the absence of helper virus infection mediate repression of transcription from the *rep* genes and from heterologous promoters placed downstream (Shi et al. 1991). This is probably an important mechanism for maintenance of latent infection. Expression of adenovirus E1A protein relieves YY1-mediated repression, and causes YY1 to further transactivate expression from the p5 promoter. The proximal YY1 binding site is located at the initiation site of the p5 promoter, and functions as an efficient initiator element either alone or downstream of TATA or SP1 binding sites (Seto et al. 1991). Synergistic enhancement of basal and E1A-induced activity has been reported *in vitro* when upstream SP1 binding sites are present, and probably occurs through direct protein-protein interaction (Seto et al. 1993). In the equivalent p6 region of the autonomous B19 parvovirus, YY1 binds to three sites and weakly transactivates the promoter, but is unaffected by E1A protein (Momoeda et al. 1994).

TR sequences themselves (145bp) have been shown to direct weak promoter activity which may arise from SP1-binding sites in the GC-rich palindromes, and from a sequence in the 'D' region bearing strong homology to an initiator (*Inr*) consensus (Flotte et al. 1993). Other putative binding elements have not been functionally defined, but are indicated (fig 5.4).

5.2.5 Helper and cellular functions in AAV DNA replication

Both helper and cellular functions are needed for efficient propagation. However, some cells become fully permissive for AAV DNA replication in the absence of helper virus if transformed and synchronised by agents such as hydroxyurea which transiently arrest cellular DNA synthesis (Yacobson et al. 1987,1989). This indicates that the function of helper is to optimise production of both AAV and cellular gene products necessary for replication. Ad DNA polymerase and terminal protein are not required, and the Ad DNA-binding protein, although serving an essential helper function, is required primarily for efficient AAV gene expression. Adenovirus helper functions are expressed from the E1A, E1B, E4, E2A and VA_I regions. E1A transactivates the p5 promoter, and

E1B and E4 promote the accumulation of AAV mRNA possibly by stabilising message, or facilitating transport to the cytoplasm. E2A DNA-binding protein and VA_I RNA may enhance translation of the p40 mRNA.

5.2.6 Latent infection by integration into the cellular genome

In the absence of helper virus the wtAAV genome integrates stably into the host cell genome by non-homologous recombination, usually in a tandem head to tail orientation. Low copy number is maintained even at high multiplicity of infection. Analysis of flanking sequences from latently infected cells has shown that integration occurs at multiple sites within a single specific locus in 60-70% of cases. The locus (AAVS1, 8.2kb) maps to human chromosome 19q13.3-qter, and has a G+C content of 82% over 900bp of flanking sequence (Kotin et al. 1992). AAV vectors in which the *rep* gene is deleted probably do not integrate at a preferential site, suggesting that *rep* gene products are important for this process. Recently, a Rep protein recognition sequence (RRS) in a 109 bp fragment of the integration locus, containing an imperfect repeating $[GCTC]_4$ motif similar to that found in the A-stem of the TR, has been shown to direct specific binding of Rep78 and Rep68, and to mediate complex formation between the AAV genome and the integration site (Weitzman et al. 1994). A putative *trs* 14bp upstream of the RRS has also been identified, and may be important for the integration process. Furthermore, when a 510bp sequence from the cloned 5' end of AAVS1 (which includes the RRS) was cloned into an episomal EBV shuttle vector, an AAV genome preferentially localised to this region (Giraud et al. 1994).

5.2.7 Adeno-associated virus-based vector systems

Based on the properties of wild-type virus, several groups have developed AAV vector systems for the efficient and stable transduction of human cells (Fig 5.5) (Hermonat and Muzyczka, 1984; Tratschin et al. 1985; Lebkowski et al. 1988). The only *cis*-acting elements necessary for rescue, replication and encapsidation of the recombinant genome are contained in the 145bp TR, which are therefore the minimal AAV sequences that are retained. Similar to adenoviruses, the maximal size for efficient packaging of the

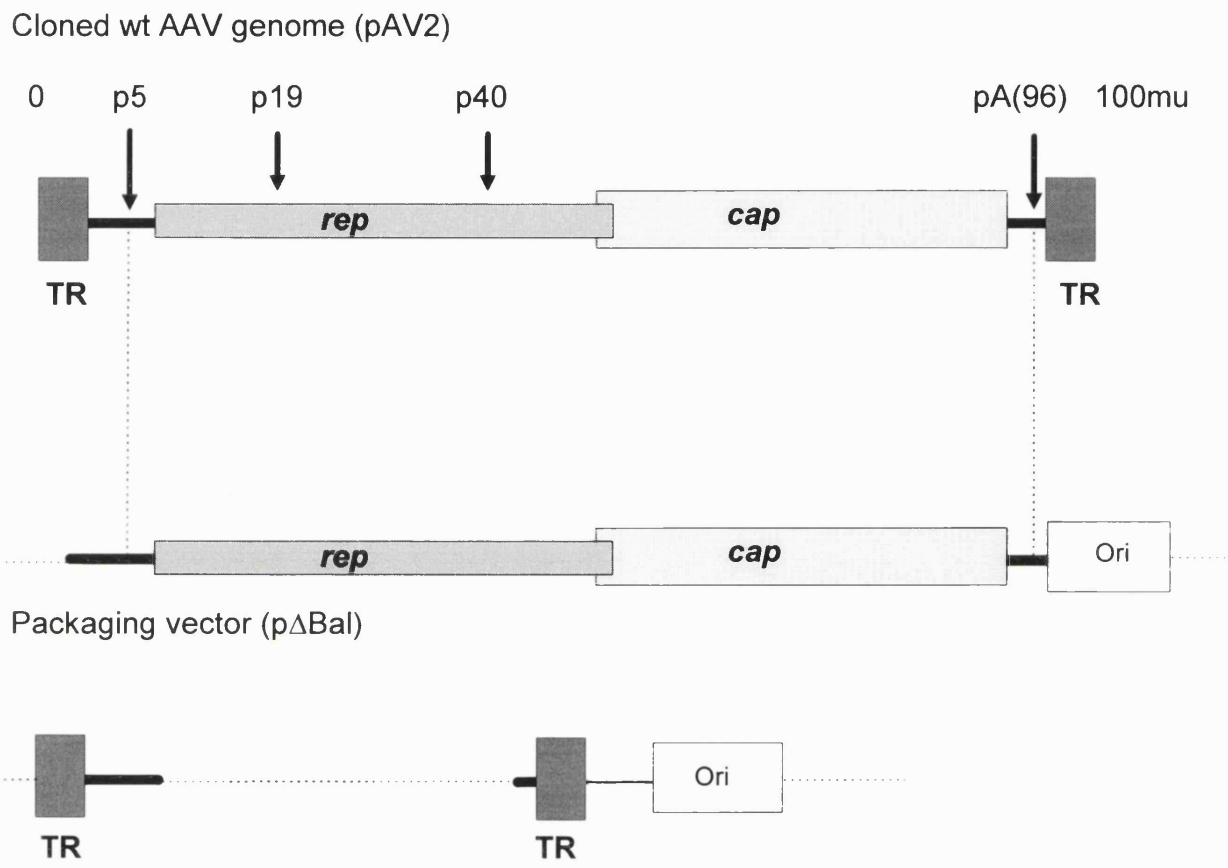


Fig 5.5. rAAV vector system (Lebkowski et al. 1988). The terminal repeats (TR) are required in *cis* for rescue from a eukaryotic genome, excision from prokaryotic plasmids, and are the minimum sequences necessary for replication, packaging and integration. These functions are therefore retained in the transducing vector. Other AAV functions necessary for replication and encapsidation are expressed *in trans* from a helper packaging plasmid, which lacks TR sequences, and which cannot be replicated. Recombinant AAV particles are generated by co-transfection of both plasmids, and simultaneous infection of a permissive cell line with helper virus, usually wild-type adenovirus.

recombinant genome is 110% of wild-type (Bett et al. 1993). Generation of recombinant viral particles is initiated by infection of a permissive cell line (usually human embryonal kidney 293 cells, or HeLa cells) with helper virus, usually wtAd5, and co-transfection of a vector genome with a separate packaging genome encoding AAV *rep* and *cap* genes. Insertion of heterologous sequence into the packaging genome to prevent encapsidation invariably lead to generation of wtAAV by recombination with the vector, or deletion. Deletion of TR sequences from the packaging plasmid reduces the levels of contamination, but also produces lower titres, possibly because the packaging genome is not replicated. A complementing plasmid in which there is no sequence homology with the vector and in which AAV coding sequences are flanked by adenoviral terminal repeats virtually eliminates the chance of wild-type contamination, and may allow some replication during helper virus infection (Samulski et al. 1989). Virus is recovered by multiple freeze-thaw cycles at completion of the cytopathic effect, and adenovirus is selectively inactivated by heating viral preparations to 56-60^{0}C for up to 1 hour, under which conditions AAV is relatively stable. Typical titres of crude unconcentrated recombinant virus achieved by these methods are around 10^4 transducing particles per ml.

The question of transduction of quiescent cells is of paramount importance for successful gene transfer to cells such as PHSCs. Recently, it has been shown that transduction frequency of S-phase cells is about 200 times that of quiescent cells, but that neither S-phase or mitosis are essential (Russell et al. 1994). Furthermore, the vector genome persisted for at least 12 days in stationary cultures, and could be recruited for transduction if the cells were induced to divide, or if treated with DNA-damaging agents (Alexander et al. 1994). Site-preferential integration may be an attractive feature of a gene transfer vector, and probably necessitates co-expression of Rep. Gene expression from vectors integrated at the chromosome 19 locus has not been investigated. Size constraints on the recombinant genome prohibit inclusion of this function in most AAV vectors, but does not exclude the possibility of co-expression *in trans* during initial transduction.

5.3 TRANSDUCTION OF IMMORTALISED CGD B CELLS BY rAAV

The following studies investigate the ability of a vector system based on adeno-associated virus to stably correct the biochemical phenotype of CGD, and identify some of the problems associated with production and purification of recombinant virus.

5.3.1 Construction of rAAV vectors

rAAV plasmids and derivatives of the vector pA1, and the packaging plasmid pΔBal are shown (Fig 5.6). pΔBal was constructed from a cloned full length AAV genome pAV2 (Samulski et al. 1982), by digestion with Ball which removes 121 nucleotides from the terminal repeats. The remaining Bgl II-linkered fragment was cloned into the BamH1 site of pUC18 (Lebkowski et al. 1988). PA1 was derived from pAV2, and retains 625 nucleotides of sequence from the left end, and 191 nucleotides from the right end of the genome. The natural polyadenylation signal at nucleotide 4420 of the full length genome is therefore deleted. In addition to the terminal repeats, the complete p5 promoter region and approximately 300 nucleotides of the left open reading frame of wtAAV are retained. This restricts the amount of heterologous material that can be inserted, and may be detrimental to expression from heterologous promoters.

The vector pAICH1 was therefore constructed by excising a 303bp Sfi1 to DraIII fragment from pA1. To compare expression of a CAT reporter gene inserted in the forward orientation under the transcriptional control of a cytomegalovirus (CMV) early promoter, vectors PA1.CMVCAT and PAICH1.CMVCAT were transfected into HeLa cells and 293 cells. Cell extracts prepared after 48 hours were measured for CAT synthesis, and to control for transfection efficiency, for co-transfected β-galactosidase activity (Fig 5.7). In HeLa cells, PAICH1.CMVCAT was shown to direct approximately 4 fold greater synthesis, although in 293 cells the difference is less marked. The explanation for this observation probably lies in the deleted portion of the p5 promoter, which includes the proximal YY1 binding site and the p5 TATA-box. The region of the promoter from −9 to +13 binds to YY1 protein, and represses transcription when

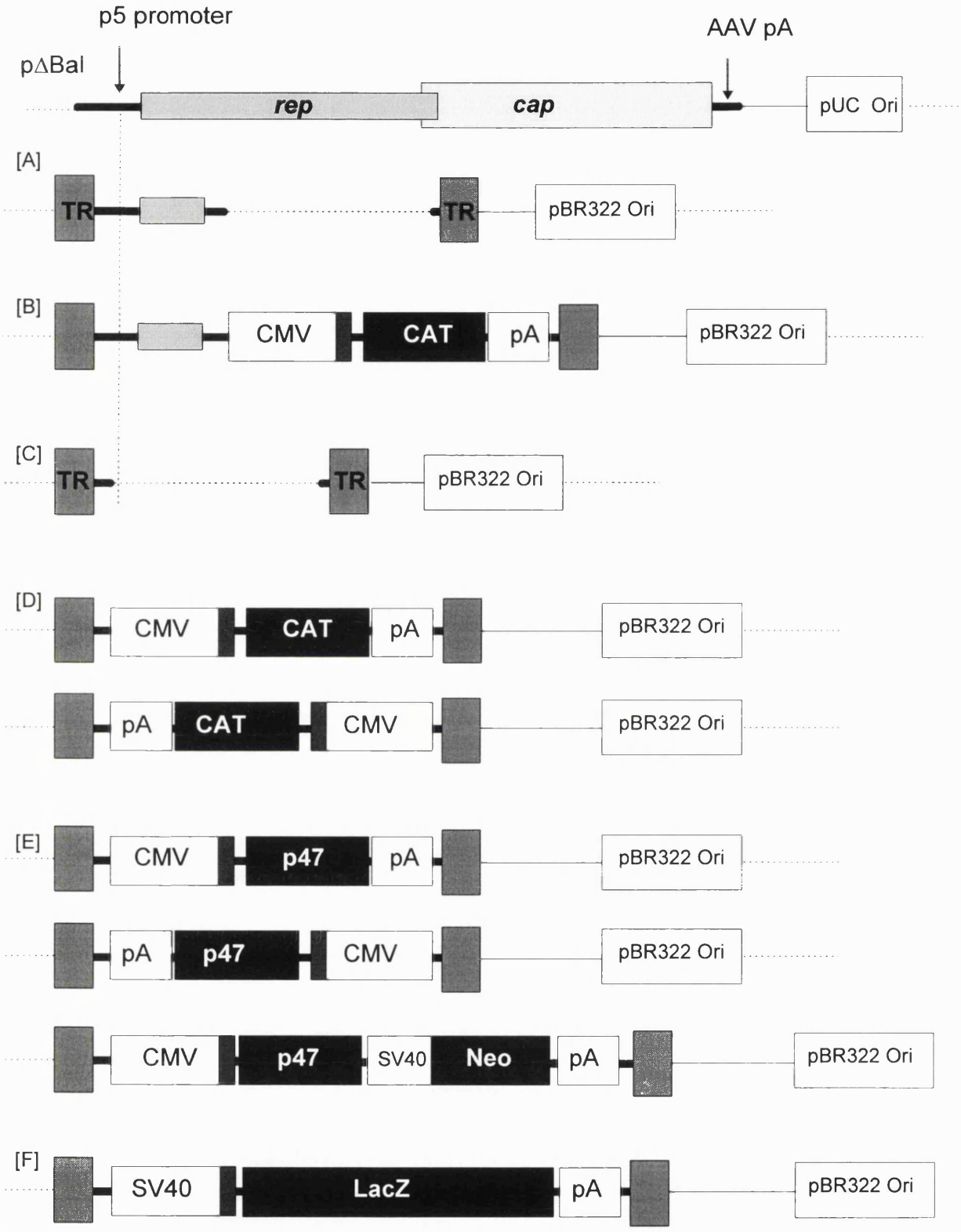
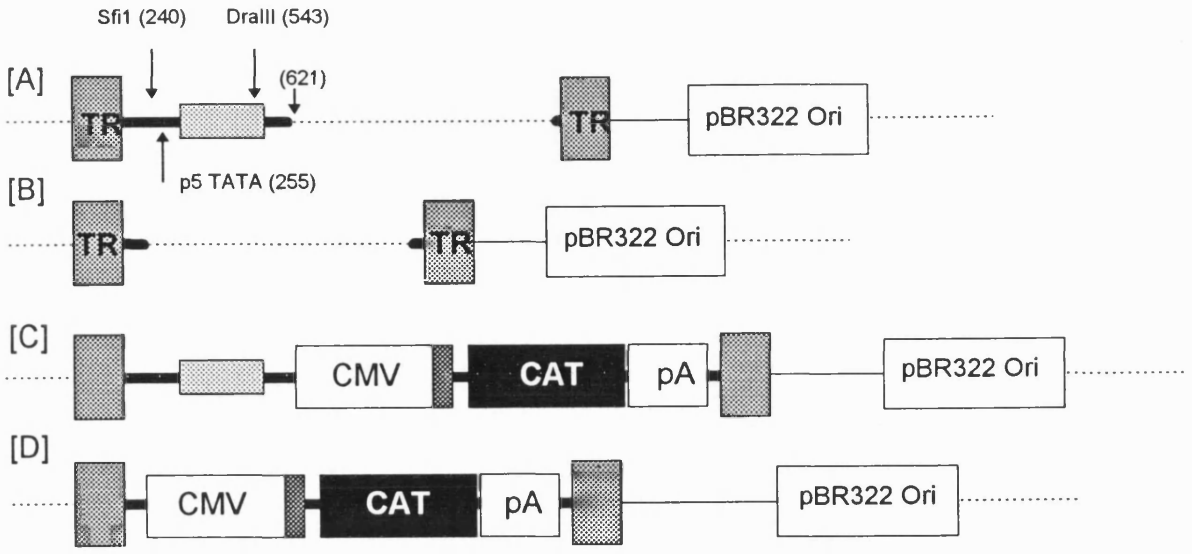


Fig 5.6. rAAV vectors. The packaging plasmid pΔBal encoding *rep* and *cap* (Lebkowski et al. 1988), and transducing vectors [A-F] are shown. For details of construction, see text. [A] PA1 (~0.81kb) Incorporates 625 nucleotides from the left end of the AAV genome, and 191 nucleotides from the right end. [B] PA1.CMVCAT (~2.9kb). [C] PAICH1 (~ 0.43kb) Incorporates 240 nucleotides from the left end, and 191 nucleotides from the right. [D] PAICH1.CMVCAT (~2.5kb), both forwards and reverse orientations. [E] PAICH1.CMV47 (~3.2kb), both forwards and reverse orientations, and PAICH1.CMV47Neo (~4.7kb). [F] PAICH1.SV40LacZ. (~4.6kb). Ori, prokaryotic origin of replication. CMV, cytomegalovirus immediate early promoter with adjacent synthetic intron. SV40, SV40 early promoter. CAT and LacZ, reporter genes encoding chloramphenicol acetyl transferase and β-galactosidase respectively. Neo, G418 resistance gene. pA, SV40 polyadenylation signal. TR, terminal repeat sequences.



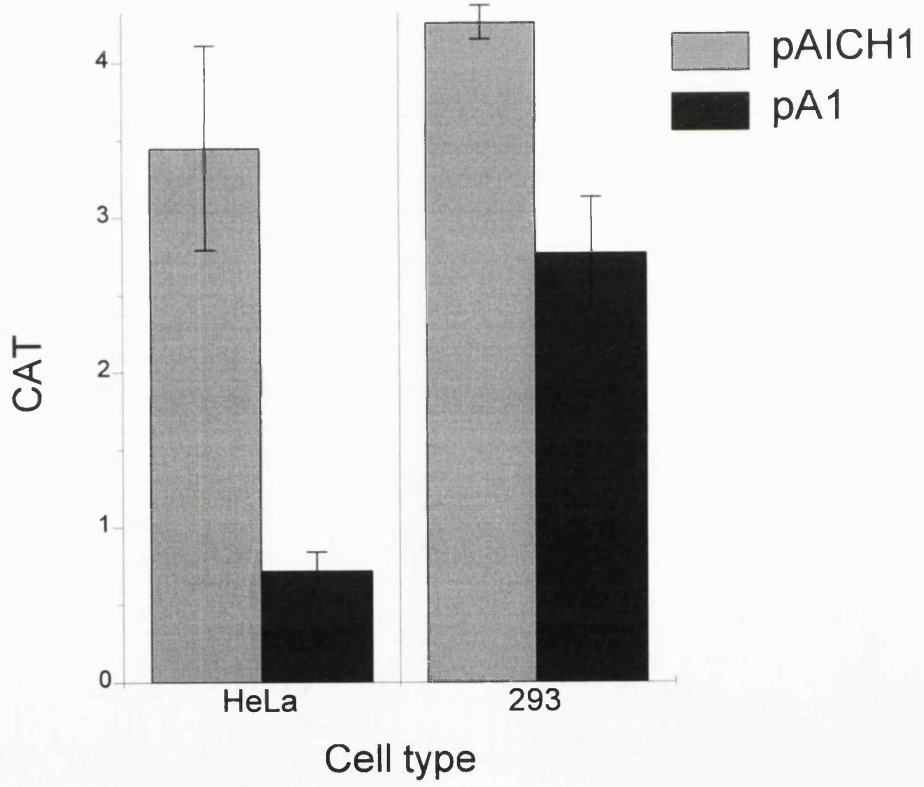


Fig 5.7. CAT activity from transfected AAV vectors. PAICH1 [B] was constructed by removal of a Sfi1 to DraIII 303bp fragment incorporating the p5 proximal promoter, and the first 222bp of the rep ORF from PA1 [A]. PAICH1.CMVCAT [D] was constructed from PA1.CMVCAT [C] by removal of the same fragment. HeLa or 293 cells were transfected by calcium phosphate co-precipitation of 10μg circular DNA, and CAT protein synthesis measured in cell extracts after 48 hours. All measurements fell within the linear range of the assay. Tranfection efficiency was determined by co-transfection of 10μg pSVβGal. CAT synthesis is measured as units of optical density (405nm), corrected for transfection efficiency, and represents the mean and standard error from three independent experiments.

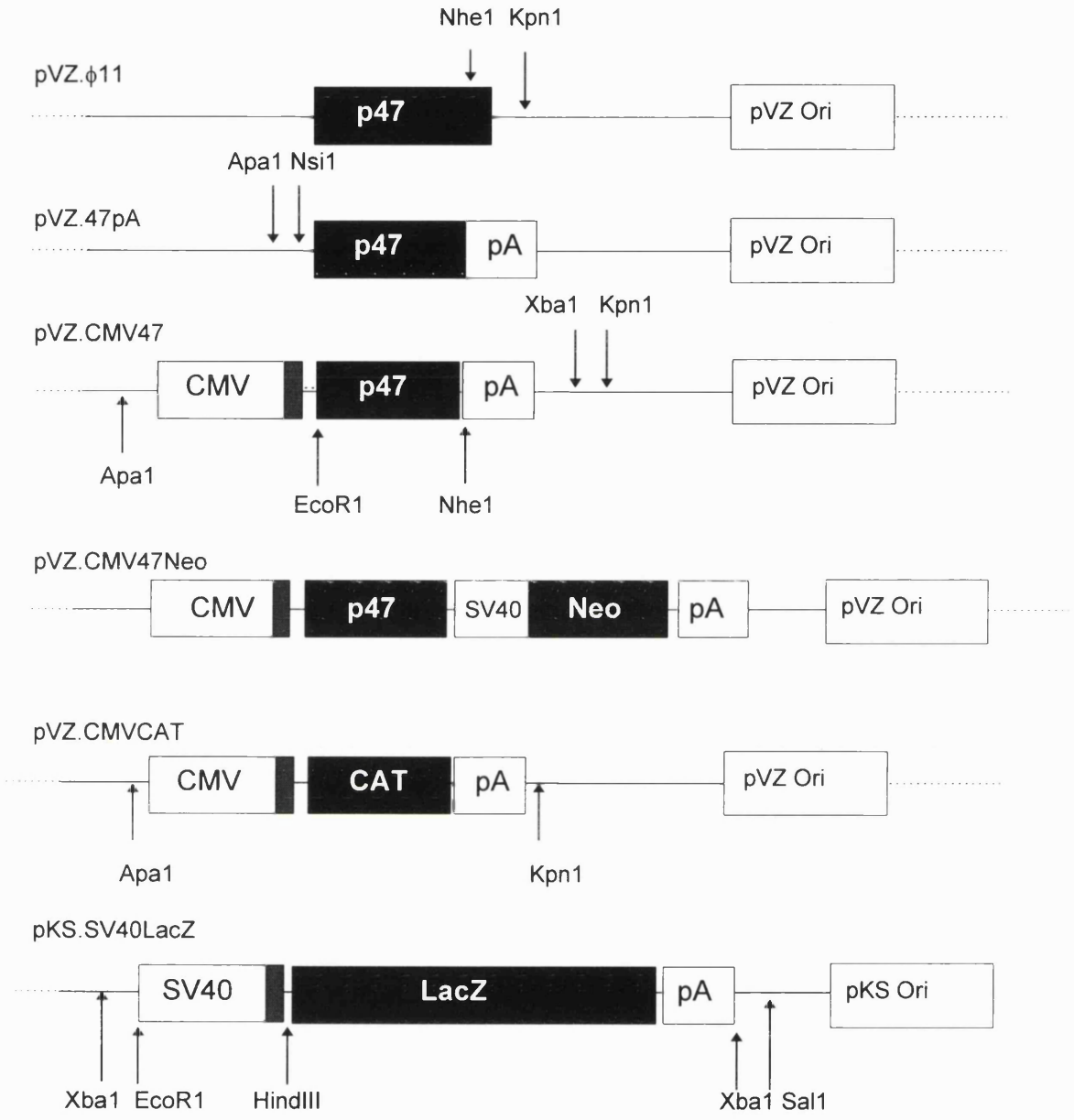


Fig 5.8. Construction of expression cassettes. A 500bp Nhe1 to Kpn1 fragment from the eukaryotic expression vector pREP8 (Invitrogen) containing an SV40 polyadenylation signal was cloned into the Nhe1 site in the 3' untranslated region of the $p47^{phox}$ cDNA, and the Kpn1 site in the polylinker. The CMV early promoter and synthetic intron were excised from PA1.CMVCAT with Apa1 and Pst1, and cloned into the Apa1 and Nsi1 sites upstream of the truncated cDNA to make pVZ.CMV47. To construct pVZ.CMV47Neo, the cDNA was excised from pVZ.CMV47 and replaced with the EcoR1 to Nhe1 fragment of pBabeNeo47 (pBN47), which incorporates the same truncated cDNA, an SV40 early promoter, a G418 resistance gene, and a prokaryotic origin of replication which was removed by digestion with Cla1. pVZ.CMVCAT was constructed from the Apa1 to Kpn1 fragment of pA1.CMVCAT, cloned directly into the complementary sites in the polylinker of pVZ. pKS.SV40LacZ was constructed in two stages. Firstly, a EcoR1 to HindIII SV40 early promoter fragment from pSVβGal (Promega) was cloned into corresponding sites in Bluescript KS (Stratagene). Secondly, the contiguous HindIII to Sal1 fragment which incorporates the LacZ gene and a polyadenylation signal was cloned into the same sites in KS to form pKS.SV40LacZ.

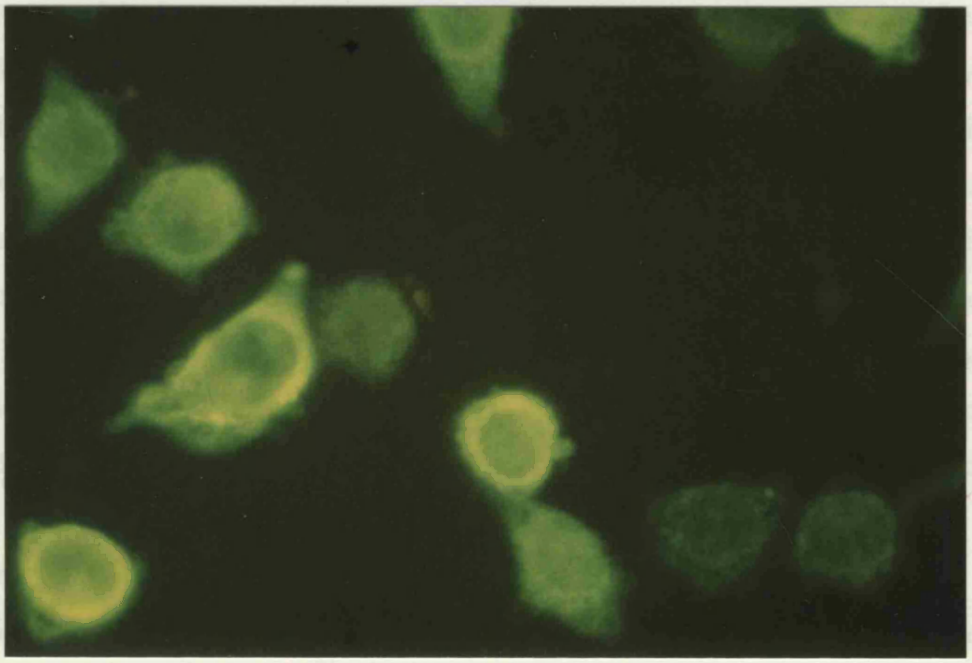
cloned upstream of a basal promoter or a complete SV40 enhancer and promoter domain (Shi et al. 1991). This repression is dependant on location of the element 5' to the promoter. Deletion of the proximal YY1 binding site in the vector probably reduces YY1-mediated repression which is most pronounced in the absence of E1A protein, and therefore diminished in 293 cells which constitutively express E1A. Deletion of the distal YY1 binding site (–50 to –72), or reversing the orientation of the expression cassette may further de-repress expression from heterologous promoters, although PAICH1.CMVCAT(R) in which the expression cassette is reversed, produces identical activity to that of the forward oriented version (not shown). It is not clear whether this observation is specific to this construct, or this promoter, but should be borne in mind when using this vector to test heterologous promoter activity.

Construction of derivatives of PAICH.1 are shown (Fig 5.6,Fig 5.8). The human cDNA encoding $p47^{phox}$, but lacking a polyadenylation signal, was cloned downstream of a CMV immediate-early enhancer and promoter, and upstream of a SV40 polyadenylation sequence in a Bluescript vector (Stratagene). The complete expression cassette was excised, and cloned into the Apa1 and Kpn1 polylinker sites of pA1 in the forward orientation. From this vector, the complete cassette was removed by digestion with Xba1, and cloned in both orientations into the modified vector PAICH1 to create the rAAV vector pAICH1.CMV47. pAICH1.SV40LacZ was created by cloning the LacZ expression cassette from pSV-β-galactosidase (Promega Corporation) into the Xba1 polylinker site of PAICH1 in the forward orientation.

5.3.2 Generation of rAAV

Recombinant AAV particles were generated by conventional methods as described in materials and methods. Briefly, 5.10^6 293 cells were plated on 9cm tissue culture dishes, and transfected the next day with 10μg each of vector and the packaging plasmid (pΔBal). 16-24 hrs later, cells were infected with adenovirus type 5 at a m.o.i. of 5-10. At completion of the cytopathic effect (48-72 hrs), cells were harvested, subjected to 4 freeze and thaw cycles, and heated to 56^0C for 30 minutes to inactivate adenovirus.

[A]



[B]

Fig 5.9. Transduction of HeLa cells by rAAV particles. Cells were incubated with heat treated rAAV supernatants for 2 hours. 24 hours later, cells were washed and tested for expression of [A] CAT (vector pAICH1.CMVCAT) by immunofluorescence or [B] β-galactosidase activity (vector pAICH1.SV40LacZ), as described in materials and methods.

Debris was separated from the virus containing supernatant by centrifugation at 1500g for 10 mins. Mock supernatant was generated in the same way except that the helper plasmid (pΔBal) was omitted from the initial transfection. For PAICH1.CMVCAT and PAICH1.SV40LacZ, virus titre was obtained by detection of CAT protein by indirect immunofluorescence, or β-galactosidase by staining, in transduced HeLa cell populations (Fig 5.9). For other vectors, a crude estimate of virus titre was obtained by comparative slot-blot hybridisation of Hirt extracted viral DNA, using virus derived from PAICH1.CMVCAT or PAICH1.SV40LacZ as standards. For all constructs, infectious titres usually approximated 10^4 particles per ml of crude supernatant, and were determined to be free from wtAAV by hybridisation to AAV *rep* gene sequences.

5.3.3 rAAV transduction of immortalised B lymphocytes

EBV-immortalised B cell lines derived from two patients with known $p47^{phox}$-deficient CGD were used to test the ability of rAAV to restore function. For these experiments, the rAAV vector pAICH1.CMV47 was used to generate a viral supernatant with an approximate titre of 10^4 transducing particles per ml. At monthly intervals post transduction, cells were harvested and assayed by chemiluminescence as described previously (Fig 5.10). Cells exposed to a mock virus preparation produced levels of superoxide indistinguishable from background obtained from activated unmanipulated cells. In contrast, $p47^{phox}$ deficient cells infected with rAAV encoding $p47^{phox}$ produced low but significant levels compared with cells derived from a normal individual. In comparison to rAAV transduced patient-derived cells, normal cells were between 50 and 100 times more active. Given the low titres of recombinant virus, and a multiplicity of infection of 0.1, transduction would therefore appear to be relatively efficient. More importantly, the degree of reconstitution remained stable for the three month period of investigation, in the absence of selection for successfully transduced cells.

To confirm the presence of immunoreactive $p47^{phox}$ in transduced cell populations, crude protein extracts from B cells derived from a normal individual, and from transduced and mock transduced $p47^{phox}$ deficient B cells, 3 months after transduction with rAAV, were reacted with specific antiserum (Fig 5.11A). Consistent with the

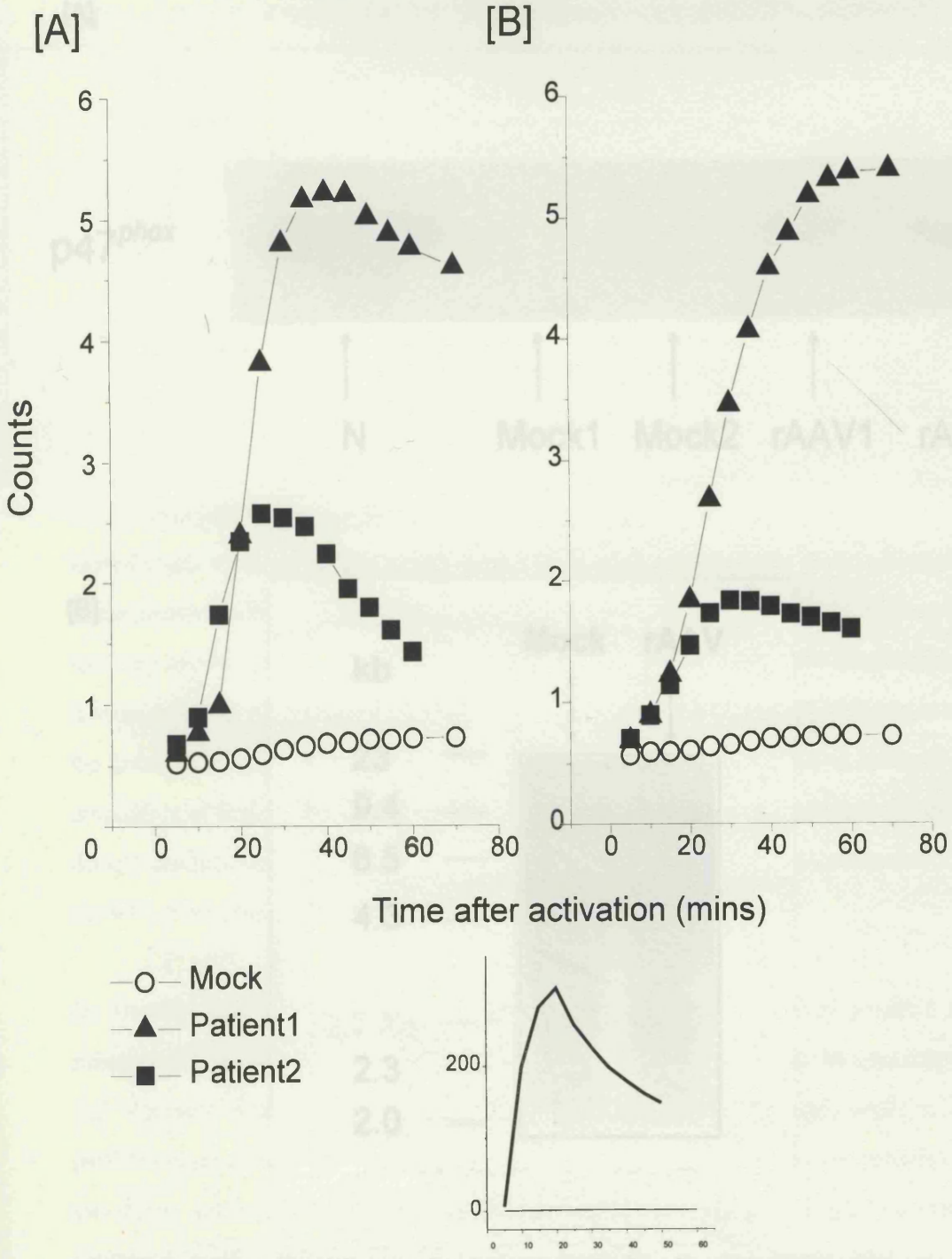


Fig 5.10. Restoration of function to immortalised B cells derived from two patients with p47^{phox}-deficient CGD. Cells were harvested and washed 2 times with phosphate buffered saline (PBS). 10^5 viable cells were resuspended in 5mls of PAICH1.CMV47 rAAV supernatant for 4 hrs, before being centrifuged at low speed, and washed once with PBS. Finally, the cells were resuspended in fresh RPMI1640 medium, and cultured for 8 weeks prior to initial analysis. As a control, the same number of cells were subjected to a sham infection using mock supernatant. Two months [A] and three months [B] post transduction, 2.10^6 viable cells were harvested and assayed for production of superoxide by chemiluminescence following stimulation with PMA. Both cell lines showed significant activity stable over time, compared to mock transduced cells. For comparison, typical responses from an equivalent number of normal and CGD cells are shown below.

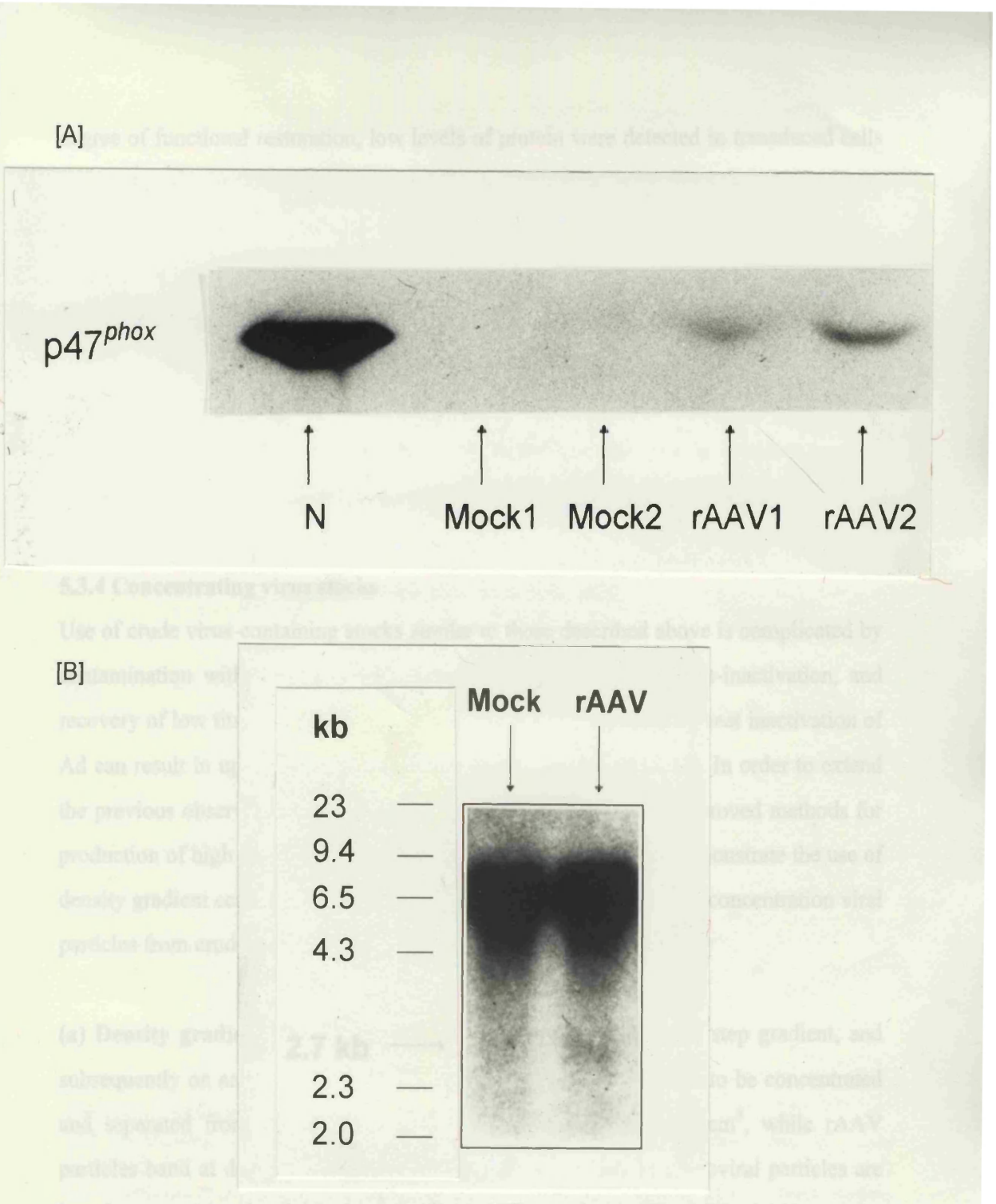


Fig 5.11. AAV-mediated transduction of CGD B cells. [A] **Western blot analysis.** Expression of $p47^{phox}$ in EBV-immortalised B cells from a normal individual (N), and cells derived from a $p47^{phox}$ -deficient patient incubated three months previously with mock supernatant (mock), or pAICH1.CMV47 rAAV particles (rAAV). Crude protein extracts from equal numbers of cells were separated by SDS-PAGE on a 12.5% polyacrylamide gel, and transferred to nitro-cellulose (Hybond C, Amersham). Blots were incubated with a polyclonal rabbit antiserum raised against the C-terminal 13 amino acids of $p47^{phox}$ as described previously, and reacting bands visualised by ECL (Amersham).
[B] **Southern blot analysis.** $p47^{phox}$ deficient immortalised B cells were exposed to mock supernatant (mock), or pAICH1.47 rAAV particles (rAAV). Genomic DNA samples (10μg) were digested overnight with Xba1 (2u/μg), separated on a 1% agarose gel, and transferred to a nylon membrane (Hybond N+, Amersham). The 2.7kb hybridising band in lane rAAV is specific to the recombinant genome.

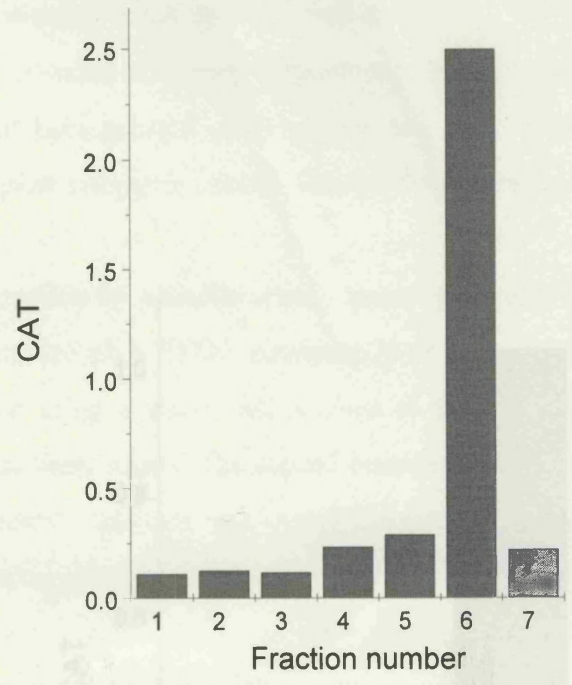
degree of functional restoration, low levels of protein were detected in transduced cells from both patients when compared to normal. In contrast, immunoreactive protein was undetectable in mock transduced cells. Genomic DNA was extracted from cells after 3 months and analysed for the presence of the rAAV genome by Southern blotting. Only DNA from functionally reconstituted cells released a predicted 2.7kb fragment which hybridised specifically to a $p47^{phox}$ cDNA probe following digestion with XbaI (Fig 5.11B). DNA from cells that underwent mock infection failed to generate this fragment under the same conditions, discounting the possibility that double stranded plasmid originating from the transducing supernatant was present in the DNA preparation.

5.3.4 Concentrating virus stocks

Use of crude virus-containing stocks similar to those described above is complicated by contamination with infectious wild-type adenovirus even after heat-inactivation, and recovery of low titres of infectious recombinant AAV. Furthermore, heat inactivation of Ad can result in up to 10-fold reduction in functional titre of rAAV. In order to extend the previous observations to primary cells, in particular PHSCs, improved methods for production of high titre virus are essential. The following studies demonstrate the use of density gradient centrifugation and ultrafiltration for purification and concentration viral particles from crude supernatants.

(a) Density gradient centrifugation: Banding of virus on a CsCl step gradient, and subsequently on an equilibrium gradient, allows the rAAV particles to be concentrated and separated from Ad. Mature wtAAV particles band at 1.41g/cm^3, while rAAV particles band at densities between this and 1.36g/cm^3. Helper adenoviral particles are less dense, and band at 1.35 g/cm^3. In the experiment shown (Fig 5.12) rAAV particles recovered from pAICH1.CMVCAT were banded on an equilibrium gradient and sequential fractions were incubated with HeLa cells without prior heat-inactivation. After 48 hours, cell extracts were tested for CAT. Maximal activity derived from fraction 6, which itself tested negative for CAT protein, and segregated from the maximal adenovirus-mediated cytopathic activity of fraction 4 which resulted in cell death after overnight incubation.

[A]



[B]

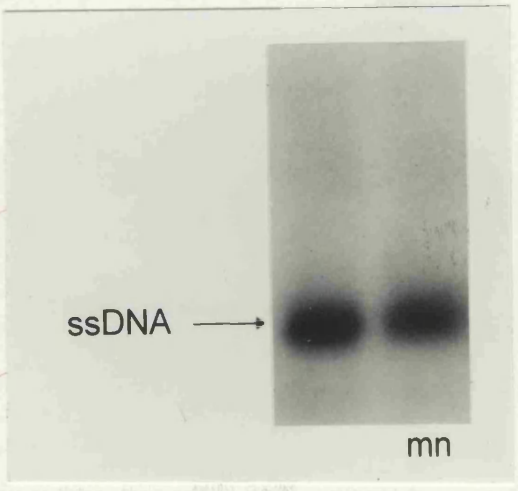


Fig 5.12. Purification of rAAV away from wt Ad.

[A] **Purification of rAAV particles by density gradient centrifugation**. 5ml of crude pAICH1.CMVCAT rAAV supernatant were layered on a CsCl step gradient (1.25g/cm^3 and 1.45g/cm^3) and centrifuged at 35,000rpm in a Beckmann SW41 Ti rotor for 1 hour at 15^{0}C. Viruses banding between the steps were retrieved by side puncture, and centrifuged at 40,000 rpm for 16 hours at 0^{0}C through a continuous CsCl gradient with an initial uniform density of 1.36g/cm^3. Fractions from this gradient were dialysed at 4^{0}C against 0.15M NaCl, and incubated with HeLa cells, which after 48 hours were tested for expression of CAT protein. Transducing activity localised to fraction 6. Adenovirus-mediated cytopathic activity was most evident in fraction 4.

[B] **Detection of ss rAAV genome in fraction 6**. DNA was purified from gradient fractions, separated on a 1% agarose gel, and blotted to a nitrocellulose membrane. The recombinant ss pAICH1.CMVCAT genome was probed with a [^{32}P]-dCTP-labelled CAT fragment. All other fractions were negative. (mn) indicates that the fraction was treated with micrococcal nuclease to digest contaminating free nucleic acid prior to disruption of intact virions.

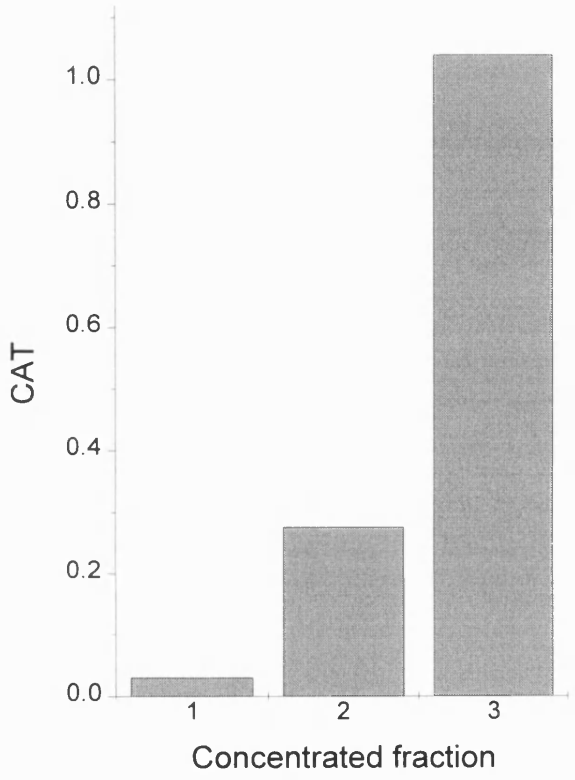


Fig 5.13. Concentration of rAAV particles by ultrafiltration. 50mls of a crude, serum-free (Optimem, Gibco) and antibiotic-free pAICH1.CMVCAT AAV supernatant (1) was subjected to ultrafiltration through a YM30 (~5nm pore size) membrane in a stirred cell at a pressure of 40psi pure nitrogen for 40 minutes. The remaining 5mls of supernatant (2) was filtered through a YM30 centriprep membrane by centrifugation at 3000g for 20 minutes. The remaining 0.5 mls of concentrated supernatant (3) was centrifuged at 13000 rpm for 30 seconds to remove debris. 200µl from each of the fractions 1, 2 and 3 was heat-inactivated and used to transduce HeLa cells. After 48 hours, cell extracts were prepared, and CAT synthesis measured as before.

The single-stranded DNA genome was detected in the same fraction treated with micrococcal nuclease to destroy contaminating non-encapsidated nucleic acid, most of which would have pelleted. After several days, cells incubated with fraction 6 also showed a typical cytopathic effect, indicating that separation was incomplete.

(b) Concentration by ultrafiltration: Concentration of a crude rAAV supernatant by ultrafiltration through a YM30 membrane (pore size ~5nm) is shown (Fig 5.13). The first filtration using a stirred cell resulted in a 10-fold increase in virus titre, with apparently minimal losses. The second concentration using a centriprep concentrator with an identical membrane was less efficient, and for a 10-fold reduction in volume, produced between 3 and 4-fold increase in transducing activity. One major disadvantage of this system is concentration of other proteins and intact adenovirus particles. A combination of ultrafiltration, followed by density gradient centrifugation should retain the advantages of both systems.

5.4 Liposomal transfection of rAAV plasmids

A recent study reported sustained and enhanced gene expression from plasmids incorporating AAV TR sequences, and it is speculated that TR sequences mediate enhanced integration of plasmid DNA in the absence of AAV translation products (Philip et al. 1994). This would overcome restrictions on genome size, and would obviate the need for encapsidation.

The ability of rAAV plasmid constructs containing vector sequences to direct efficient and sustained gene expression in different cell types was therefore investigated. The plasmid constructs PVZ.CMVCAT and PA1CMVCAT contain identical CAT expression cassettes, which in PA1.CMVCAT is also flanked by AAV TR sequences. These were transfected as complexes with cationic liposomes into 293 cells, HeLa cells, and EBV-immortalised B cells. In all three cases CAT expression had disappeared by 2 to 3 weeks (Fig 5.14). Retention of AAV sequences in the vector therefore failed to confer stability to transduction, and produced initial levels of expression no higher than those obtained with the basic expression vector. In HeLa cells, initial expression was considerably lower, presumably due to a repressive influence on transcription mediated

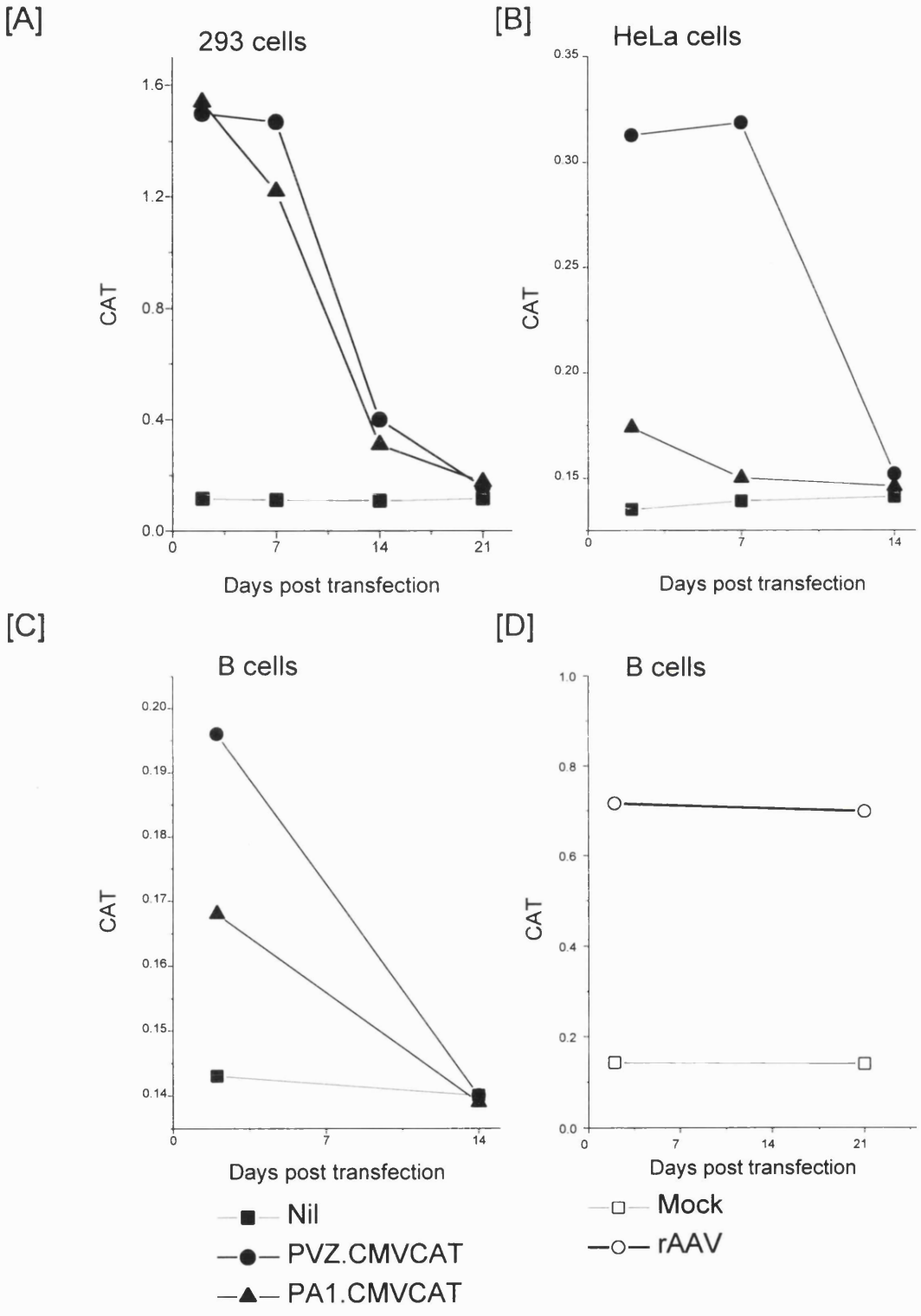


Fig 5.14. Stability of expression from transfected AAV plasmids. PVZ.CMVCAT and PA1.CMVCAT differ primarily with respect to AAV TR sequences in PA1.CMVCAT which flank an identical CAT expression cassette to that of PVZ.CMVCAT. These were transfected as liposomal complexes into [A] 293 cells, [B] HeLa cells and [C] EBV immortalised B cells [C]. CAT expression was measured at 48 hours post transfection, and at varying weekly intervals afterwards. Retention of TR sequences conferred no additional stability to the transfection. These results are contrasted with sustained expression in EBV-immortalised B cells infected with recombinant PA1.CMVCAT AAV particles [D].

by YY1-binding sequences, both of which are retained in PA1.CMVCAT. In contrast, B cells infected with PA1.CMVCAT-derived rAAV particles showed sustained expression of CAT for at least 3 weeks. In contrast to published observations (Philip et al. 1994), incorporation of AAV TR sequences would therefore appear not to confer any benefit to transduction stability or gene expression from a plasmid vector.

5.5 DISCUSSION

To date, the most widely applied vector system to haematopoietic gene therapy is based on murine leukaemia retroviruses, but is limited by an inability to efficiently transduce the relatively quiescent PHSC population. This deficiency reflects a block to nuclear entry of the transducing genome imposed by the nuclear membrane, which is overcome at the time of cell division. Strategies directed towards stimulation of stem cell proliferation, or development of gene transfer systems based on viruses such as human immunodeficiency virus (HIV) which encode specific nuclear localisation signals may overcome this problem, but are not currently applicable. Vectors systems based on the human parvovirus, adeno-associated virus-2 (AAV-2) have many attributes that recommend their use for therapeutic gene transfer, including a broad tissue tropism, stable integration into the host genome, and transduction of quiescent cells.

These studies are the first to develop rAAV vectors for gene therapy of CGD, and demonstrate that cells derived from patients can be efficiently and stably transduced in the absence of selection. Although not specifically investigated, random integration of the recombinant genome is likely in view of stability of expression with multiple cell passage. However, persistence of episomal copies has not been excluded, and has been described in other situations.

Conventional methods for generation of rAAV particles produce relatively low titre viral stocks, which are invariably contaminated with wtAd. At present this restricts the evaluation and application of this vector system to cultured cells. Concentration and purification of rAAV using ultrafiltration and density gradient centrifugation is partially effective, and in combination can achieve transducing titres of 10^5-10^6 per ml, although

adenovirus is never fully eliminated. Improved methods for recovery of virus from cells using proteolytic or detergent-based extraction methods, together with chromatographic depletion of adenoviral particles, or fractionation of rAAV with specific antibody may further improve the purification process.

Cell delivery of the unpackaged rAAV genome by alternative means, for example in a complex with cationic liposomes, would overcome the restrictions on genome size, and would allow incorporation of sequences encoding *rep* in the vector. Unfortunately production of single-stranded, or 'no-end' double stranded DNA species which mimic the secondary structure of the AAV genome has proved difficult (Nahreini et al. 1992). Contrary to previous reports, liposome-mediated delivery of rAAV vectors in the form of double stranded DNA plasmids confers no benefit to transduction efficiency or stability, at least in the absence of Rep protein.

Optimisation of methods for production of purified recombinant virus particles is essential before the full potential of rAAV gene transfer can be evaluated.

ADDENDUM

The titre of AAV viral preparations can be determined by quantitative detection of the encapsidated recombinant genome, or by detection of the recombinant genome or products expressed from this genome in infected cells. In general, the particle number is 1000-fold greater than the infectious titre determined by transduction of either HeLa or 293 HEK cells in culture.

Uptake of recombinant protein present in unpurified crude AAV preparations can result in false positive transduction events. However, for the experiments described, the persistence of expression observed in infected cells most probably reflects true gene transduction events, and is supported by the co-incident detection of recombinant forms of the $p47^{phox}$ gene.

Latent infection by AAV may involve persistence of the genome in an episomal form, as well as integrated into host chromosomal DNA. The efficiency of integration of recombinant AAV genomes is at present unknown, but is likely to be low. The contribution from either form following transduction of immortalised CGD B cells has subsequently been investigated in polyclonal populations, and suggests that the recombinant genome is integrated.

5.6 SUMMARY: ADENO-ASSOCIATED VIRUS GENE TRANSFER FOR CGD

- *rAAV has been shown to transduce immortalised B-cells derived from CGD patients. Integration of the rAAV genome into host chromosomal DNA is inferred from the stability of transduction.*

- *The levels of transduction are low, primarily reflecting difficulties in production of large quantities of recombinant virus.*

- *Titres of recombinant virus can be enhanced by concentration using ultrafiltration, and by density gradient centrifugation. The latter technique is partially effective in separating contaminating adenovirus.*

- *Transduction of target cells using TR-containing double stranded templates, at least in the absence of Rep expression, does not confer stability.*

CHAPTER 6:

FACILITATING RECOVERY OF rAAV PARTICLES

6.1 INTRODUCTION

The low levels of transduction seen in the previous experiments, and in those reported by other groups, are primarily a reflection of inefficient methods available for production of rAAV. Conventionally, propagation of rAAV is initiated by infection of producer cells with wtAd5, and expression of the early gene products necessary for AAV replication (E1a, E1b, E2a, E4 and VA_I), while AAV Rep and Cap functions are expressed from a helper plasmid which is co-transfected with the corresponding vector plasmid. Each cell successfully transduced by all three components may yield up to 10^4 viral particles, but the general inefficiency of the transfection process means that only a small percentage of potential producer cells propagate recombinant virus. Concentration of viral particles by physical means is helpful, but is complicated by a requirement for large quantities of starting material to achieve significant increases in viral titre. Furthermore, heat treated rAAV stocks are contaminated with low levels of infectious wt Ad.

6.2 FACILITATING PRODUCTION OF rAAV PARTICLES

Enhanced delivery of the vector genome to cells in which rAAV is propagated may allow recovery of higher titres, simply by increasing the number of productive cells. While physical methods for transfection of cells, such as calcium phosphate precipitation, are relatively inefficient, vectors based on adenovirus achieve high titres and transduce a wide variety of cell types with high efficiency (Berkner, 1992). Utilisation of these positive features in the production of rAAV stocks free from wild-type virus may therefore be beneficial.

6.2.1 Adenovirus life cycle

Adenoviruses are non-enveloped icosohedral double-stranded DNA viruses which usually produce relatively minor upper respiratory tract and gastrointestinal infections

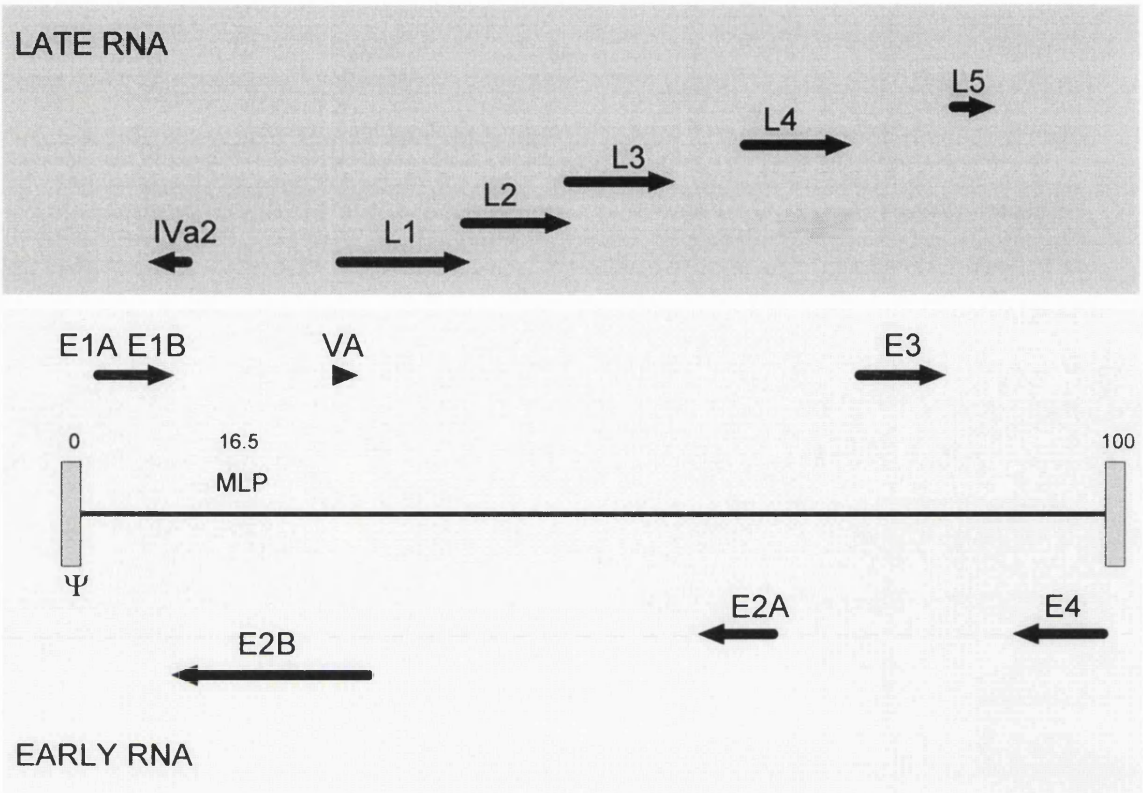


Fig 6.1. Simplified representation of the adenovirus genome. ORF's of transcripts are shown for the non-structural early genes (E1-E4), the late genes that encode structural elements (L1 to L5, IVa2), and a RNA polymerase III transcript (VA). The early genes each have individual promoters, while the majority of late genes are transcribed from a single major late promoter (MLP). Flanking boxes represent inverted terminal repeat sequences (ITR) which contain all the cis-acting elements necessary for replication and encapsidation of the genome. The packaging signal is represented by Ψ.

in humans (Horwitz, 1990). The adenovirus growth cycle is comprised of two phases which overlap one another. In the early phase before the onset of DNA replication, cellular metabolism is subverted for lytic replication in permissive cells, or transformation of non-permissive cells (Fig 6.1). In the late phase, structural proteins are synthesized and mature virions assembled. The arrangement of the 36kb genome in over 40 serotypes is similar, although the most extensively mapped are those for adenovirus 2 and 5. Overlapping families of viral transcripts from nine promoters are co-terminal at their 3', 5' or both ends. Virtually all late genes are represented by 5 families of 'r' strand mRNA (L1 to L5) derived from the major late promoter (MLP) at map position 16.5. Early genes are transcribed from six different promoter sites. mRNA is synthesized by cellular RNA polymerase II, in addition to VA_I RNA and VA_{II} RNA which are synthesized by RNA polymerase III.

The key determinant of host cell tropism is virus attachment to specific cell-surface receptors (White, 1993). Infection is initiated by virus-binding to an uncharacterised receptor by coat fibre protein. Following attachment, internalisation occurs by receptor-mediated endocytosis, an event which is mediated by Arg-Gly-Asp (RGD) peptide sequences in virus penton base coat protein, identical to those found in cell matrix adhesion molecules such as fibronectin which bind to cell surface α_v integrin. This step is distinct and dissociable from fiber-mediated attachment (Wickham et al. 1993). Tropism is therefore not only dependant on the relative amounts of fiber and penton base receptors on different cells, but on the occupancy of α_v integrins by cell matrix protein. Acidification of the endosome results in disruption, allowing virions to proceed to the nucleus to complete their life cycle. Endosomal disruption is independent of viral gene expression, and is a direct function of virus coat protein. Once in the nucleus, replication is initiated by a viral protein covalently linked to the 3' end of both strands of the genome.

6.2.2 Adenovirus-based vector systems

The ability to achieve high titres of relatively stable virus (10^9-10^{12} infectious particles per ml), broad tissue tropism, and efficient infection of non-dividing cells are attractive features for a gene transfer system. The relative safety of adenoviral vaccines (serotypes

4 and 7) and vectors (serotypes 2 and 5) has resulted in several human gene therapy applications, particularly for *in vivo* delivery of genetic material. In contrast to retroviruses, the adenoviral genome integrates rarely into host chromosomal DNA, and in the absence of continuing infection, is not transmitted to progeny cells. This is a limitation for curative therapy of inherited haematopoietic disorders, but may be ideal for situations in which transient expression is desirable.

Use of adenoviruses as replication-defective gene transfer vehicles first became possible when it was shown that viruses lacking parts of the E1 region could be propagated in cells engineered to complement the E1 deficiency (Jones and Shenk, 1979). Subsequently it was shown that adenoviruses containing heterologous genetic material could be propagated in the same way. The first generation of adenoviral vectors contained deletions in the E1 and/or the E3 regions of the genome. E1 proteins are important transcriptional activators of both viral and cellular genes, and recombinant virus deleted for these functions must be propagated in human embryonal kidney cells (293 cells) which were transformed by transfected sheared adenovirus DNA, and which contain 4-5 copies of the left end 12% and 1 copy of the right end 10% of the full length genome (Graham et al. 1977). Production of similar producer cell lines has been complicated by the toxicity of E1 proteins. The E3 region is important for subversion of host immune response, but is unnecessary for viral propagation. Packaging efficiency of a recombinant genome is dramatically reduced if size exceeds 105 % of the length of wild-type, so that vectors in which both E1 and E3 regions are deleted can accommodate up to 8Kb of heterologous material (Bett et al. 1993). Recombinant virus is produced either by direct cloning into a deleted full length genome, or by homologous recombination between a partial shuttle genome containing the deletion, and a full length genome rendered un-packageble by the incorporation of heterologous sequences (Haj-Ahmad and Graham, 1986).

The requirement for E1 expression is not absolute, and persistent replication of E1-deleted vectors can occur in the absence of this function, particularly at high multiplicity of infection (Jones and Shenk, 1979; Mitani et al. 1994). In some cases, host cellular or viral proteins may be able to complement E1 function (Imperiale et al. 1984).

Furthermore, retention of large amounts of the adenoviral genome in the vector molecule leads to direct cellular toxicity, and induction of host immunity (Yang et al. 1994). In the latter case, deletion of the E3 region appears to enhance immunogenicity, a process which in the absence of cell division is largely responsible for transient expression of heterologous genes. Second generation vectors are currently under investigation in which additional regions of the adenoviral genome are inactivated in the recombinant virus. These are potentially less toxic, less immunogenic, and can incorporate additional genetic material. Examples of second generation vectors include those derived from a mutant virus strain with a temperature sensitive mutation in the E2 region, which permits propagation at low temperature, and reduced expression of E2 protein at 37^0C (Engelhardt et al. 1994), and vectors deleted for E4 which itself is a potent transactivator of E2A and the immunogenic late proteins. Complementary packaging cell lines for vectors deleted at other loci are under development, but are restricted by toxicity of both early and late proteins. Minimal vectors in which the only retained adenoviral sequences are those required in *cis* for encapsidation and replication of the recombinant genome are under investigation, but at present can only be rescued at low titre.

6.3 RESCUE OF rAAV FROM AN ADENOVIRAL VECTOR GENOME

These studies demonstrate that rAAV particles can be efficiently rescued and amplified from an E1-deleted adenoviral vector which incorporates AAV vector sequences. In complementary cell lines this obviates the need for wtAd.

6.3.1 Construction of a recombinant adenoviruses (rAD)

Residual AAV sequences, which include both TRs, were completely excised from PAICH1, and cloned into the corresponding polylinker sites of the left end E1-deleted (deletion from 452bp to 3328bp) adenoviral plasmid pXCJL.1 (McGrory et al. 1988) to form pXCJL.ITR (Fig 6.2). A complete $p47^{phox}$ expression cassette was cloned into pXCJL.ITR in the forward orientation, to create the hybrid rAd/rAAV vector pXCJL.ITR47. The corresponding replication-defective recombinant adenovirus was rescued by homologous recombination with a co-transfected full length adenovirus

genome pJM17 (McGrory et al. 1988) in E1-complementary 293 cells. pJM17 contains an insertion of pBR322 derivative at bp 1339 which makes this genome too large to package. Plaque isolated clones were amplified on 293 cells, and the predicted genomic organisation of the recombinant viruses confirmed by Southern blot analysis of purified viral DNA (not shown). AAV sequences remained stable within the adenoviral vector genome during propagation. The viral titre of supernatants obtained from 5 clones (p47AD1-5) was estimated by cytopathic end-point assay as described in materials and methods. From one selected individual clone (p47AD2) the titre of supernatant was determined to be approximately 2.10^9 pfu/ml by conventional plaque assay. p47AD2 stocks were used for all experiments. To ensure that amplified viral stocks were free from wtAd, HeLa cells were infected with recombinant adenovirus, and observed for cytopathic effect (CPE). At the end of a 2 week period, no such effect was observed. A parallel E1-deleted adenoviral vector encoding *rep* and *cap* but lacking AAV TR sequences was constructed as shown, but could not be rescued by recombination in 293 cells (Fig 6.2).

6.3.2 Transduction of EBV-immortalised B cells by recombinant adenovirus.

An EBV-immortalised B cell line derived from a patient with known $p47^{phox}$ deficient CGD was used to test the ability of the recombinant adenovirus p47AD2 to restore NADPH-oxidase function (Fig 6.3). For these experiments, 5.10^6 viable cells were washed twice with PBS, and incubated with p47AD2 in phosphate buffered saline (PBS) at 37^0C, m.o.i. 100 and 10. After 2hrs, cells were washed and refed with fresh medium. 48hrs and 14 days later, 2.10^6 cells were removed from culture and tested for their ability to produce superoxide as described previously. Although function was efficiently restored to these cells at levels determined by the m.o.i, in all cases activity was undetectable at 2 weeks, reflecting the very low frequency at which the adenoviral genome integrates into the host genome, and the inability for the genome to be transmitted to progeny cells in the absence of continuing viral replication and infection. The absence of sustained expression from this vector is further support for previous studies in which retention of TR sequences within double stranded plasmid vectors did not confer stability to transduction.

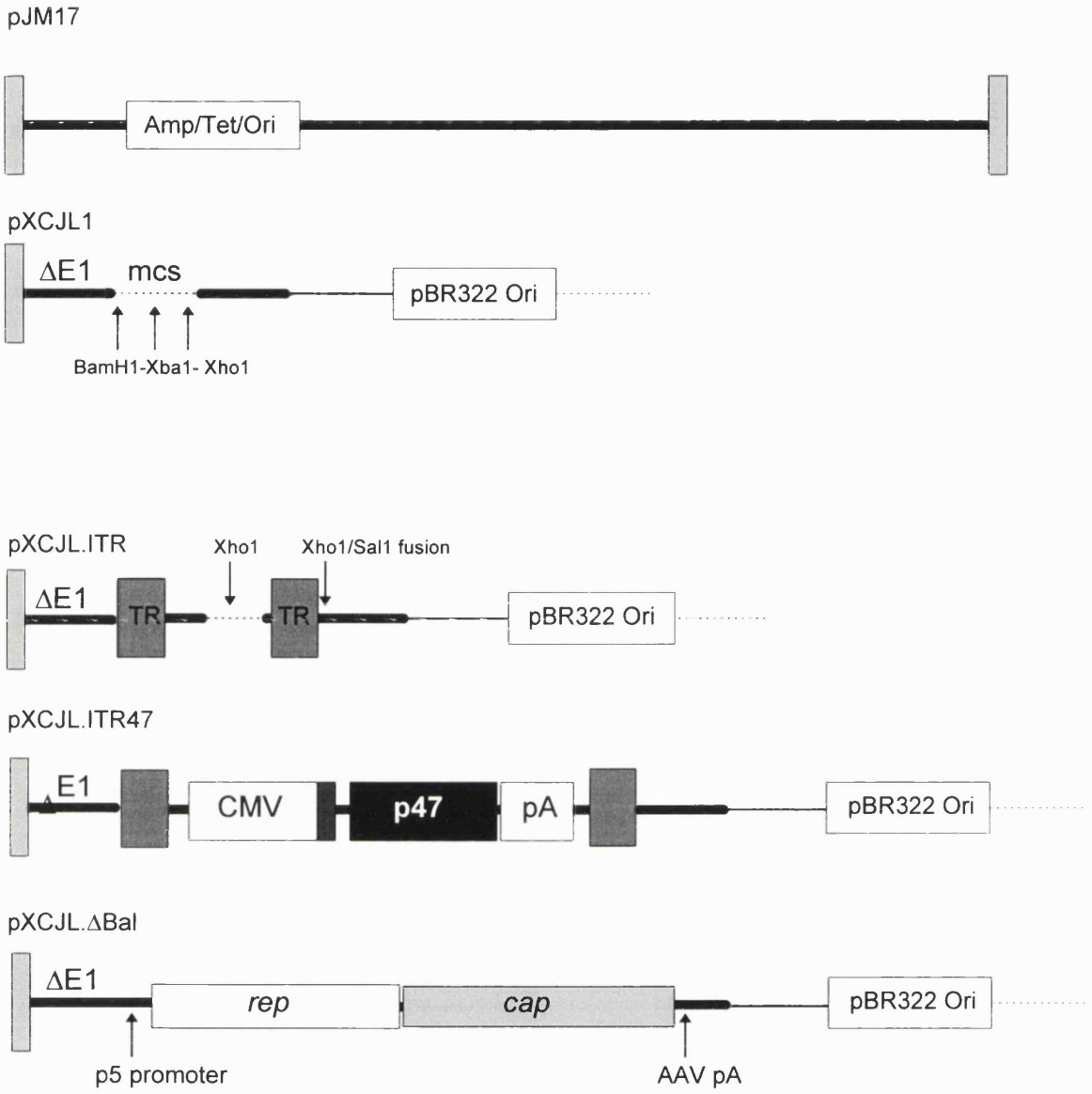


Fig 6.2. Construction of adenoviral vectors. The parent left-end E1-deleted (ΔE1) adenoviral vector pXCJL1 is shown below a complementary full length adenoviral genome, pJM17, in which an inserted pBR322 derivative encoding resistance to ampicillin and tetracycline (Amp/Tet) and prokaryotic origin of replication (Ori), exceeds the packaging size limit for adenovirus. pXCJL.ITR was constructed by isolation of both AAV ITR sequences (TR) from pAICH1 by digestion with BamH1 and Sal1, and cloning this into the BamH1 and Xho1 sites in the multiple cloning site (mcs) of pXCJL1. A complete expression cassette for $p47^{phox}$ was excised from pAICH1.CMV47 by digestion with Xba1, blunted and directly cloned into a blunted unique Xho1 site in pXCJL.ITR in the forward orientation. The replication-defective AAV genome was removed from the pUC18 backbone of pΔBal by digestion with Xba1 and Sma1, and cloned into complementary Xba1 and blunted Xho1 sites in the multiple cloning site (mcs) of pXCJL1 to make pXCJLΔBal. Recombinant viruses were rescued by co-transfection with pJM17 in E1-complementary human embryonal kidney 293 cells.

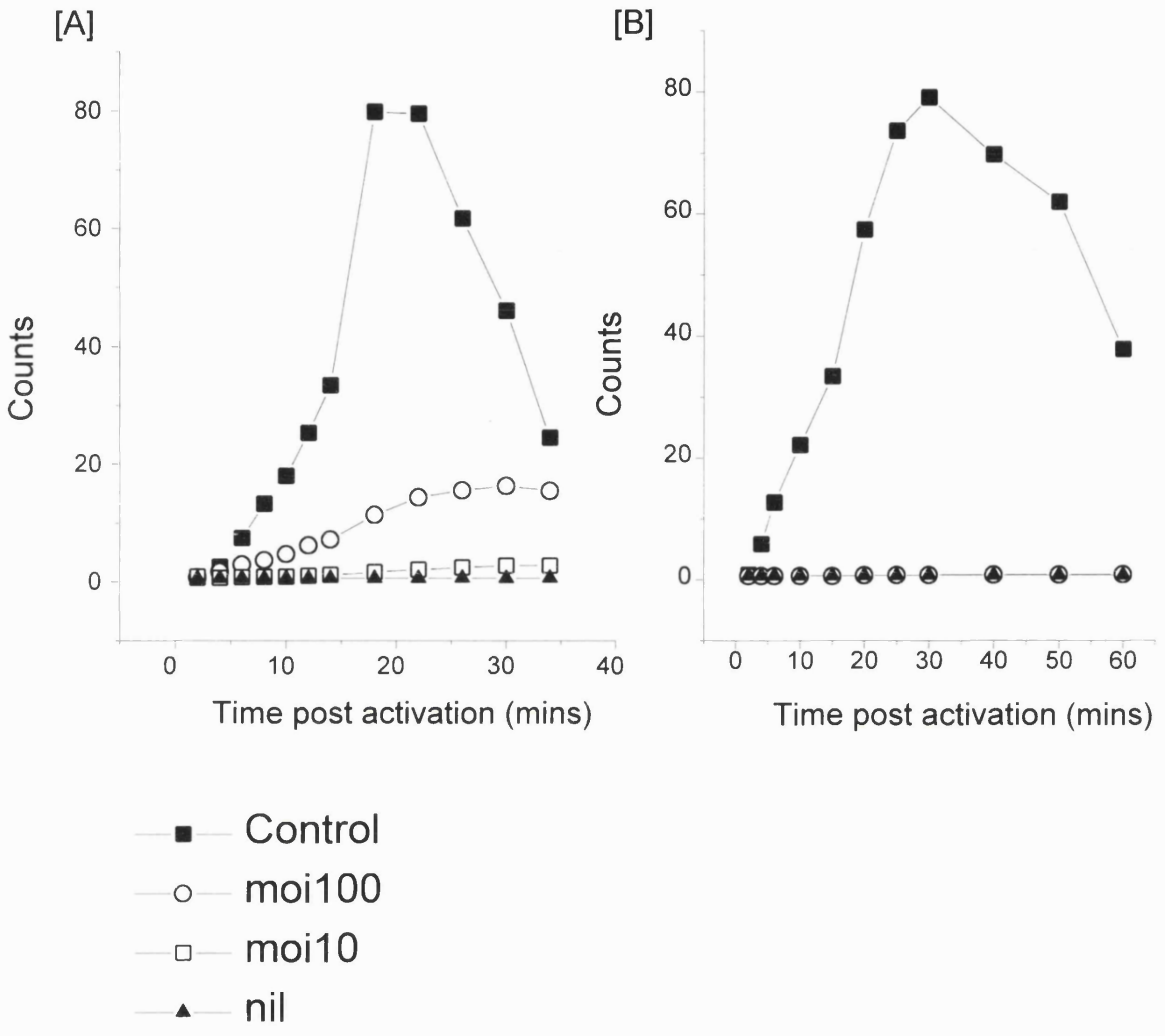


Fig 6.3. Restoration of function to p47^{phox}-deficient immortalised B cells by a recombinant adenoviral vector. 5.10^6 cells were incubated with p47AD2 recombinant adenoviral particles at a m.o.i. of 10 and 100 for four hours, and replaced in culture. 48 hours [A] and 2 weeks [B] post transduction, 2.10^6 viable cells were harvested and assayed for production of superoxide by chemiluminescence following stimulation with PMA. Significant restoration of function was observed in a dose-related manner, but as predicted from the nature of the transducing vector, was not sustained over time. Control and nil represent unmanipulated cells derived from a normal individual and a patient respectively.

6.3.3 Production of rAAV

The rAAV genome was rescued and amplified from the adenoviral vector by the following method. 5.10^6 293 cells were plated on 9cm tissue culture dishes, and 16-24hrs later infected with p47AD2 at a m.o.i. of 5-10. After a further 2-4hrs, cells were transfected with 10μg of packaging plasmid (pΔBal), by calcium phosphate co-precipitation. 48-72hrs later, at completion of the cytopathic effect, cells were harvested, and subjected to 4 freeze and thaw cycles. Debris was separated from the virus containing supernatant by centrifugation at 3000g for 10 mins. One half of the virus containing supernatant was heated to 56°C for 20 minutes to inactivate recombinant adenovirus. Mock supernatant was generated in the same way except that the packaging plasmid (pΔBal) was omitted from the transfection. Supernatants were analysed by Hirt extraction of low molecular weight DNA (Fig 6.4). In the presence of helper plasmid, both rAAV and rAd replicative species were efficiently rescued and amplified. In contrast, when neutral DNA was transfected instead of the packaging plasmid pΔBal, rescue and replication of rAAV did not occur at detectable levels, although replication of the adenoviral vector occurred efficiently. Titre of rescued rAAV was estimated to be ~ 5.10^4 transducing particles per ml, determined by slot blot hybridisation, and was shown to be free from wtAAV by probing for *rep* gene sequences (not shown).

6.3.4 rAAV transduction of immortalised B lymphocytes.

An EBV-immortalised B cell line derived from a patient with known $p47^{phox}$ deficient CGD was used to test the ability of rAAV rescued from p47AD2 to restore NADPH-oxidase function (Fig 6.5). Cells were harvested and washed 3 times with PBS. 10^5 viable cells were resuspended in 2mls of both heat-treated, and untreated rAAV supernatant for 4hrs, before being centrifuged at low speed, washed and resuspended in fresh medium. As a control, the same number of cells were subjected to a sham infection using mock supernatant. In contrast to B cells transduced by p47AD2 alone, those cells exposed to supernatants containing both p47AD2 and rAAV rescued from p47AD2, maintained detectable levels of superoxide production at least to 8 weeks post transduction. To control for persistence of the adenoviral vector, cells which were transduced with the same rAAV supernatant which had been heat treated to selectively

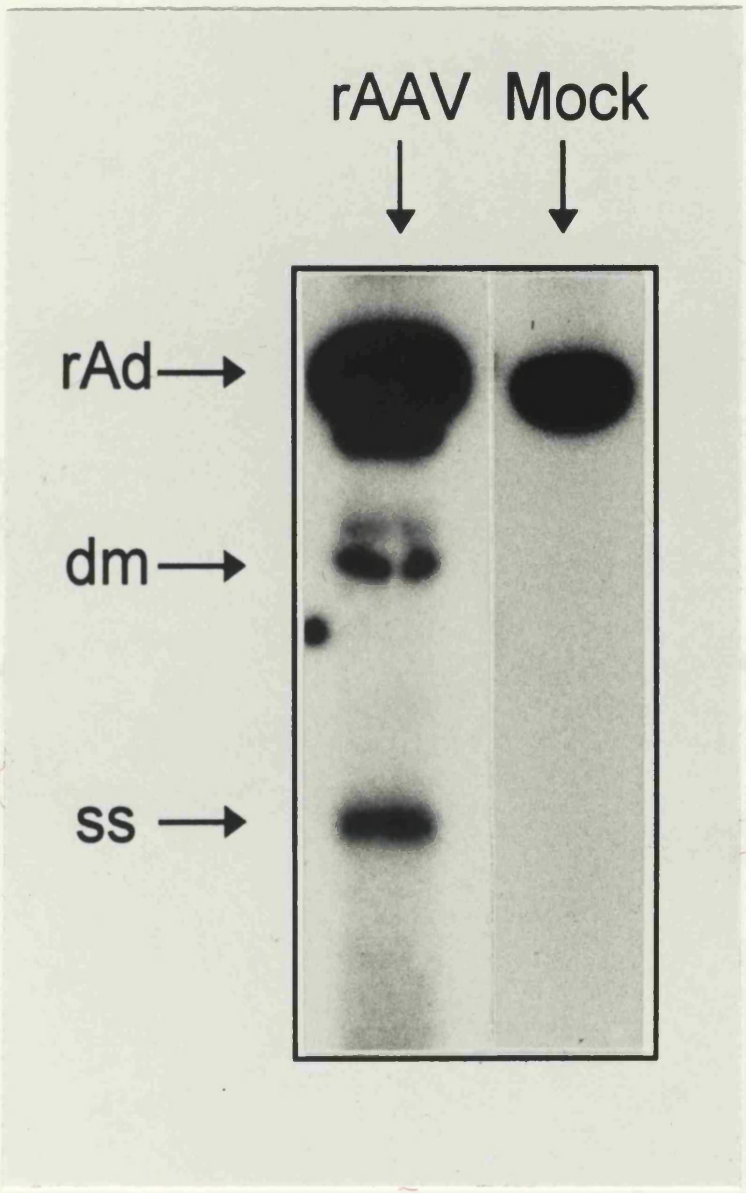


Fig 6.4. Replication and rescue of rAAV in 293 cell lines infected with recombinant adenovirus incorporating rAAV vector sequences. All cells were infected with p47AD2 at a m.o.i of 5-10. In the lane marked rAAV, cells were co-transfected with the AAV packaging plasmid pΔBal. In the mock experiment, this step was omitted. At completion of the cytopathic effect, low molecular weight DNA was isolated by Hirt extraction, and analysed by Southern hybridisation with a $p47^{phox}$ cDNA probe. Although recombinant adenovirus replicates efficiently in both situations (rAd), only those cells co-transfected with the AAV packaging plasmid permit rescue and amplification of duplex monomeric (dm) and single stranded progeny (ss) species of the rAAV molecule.

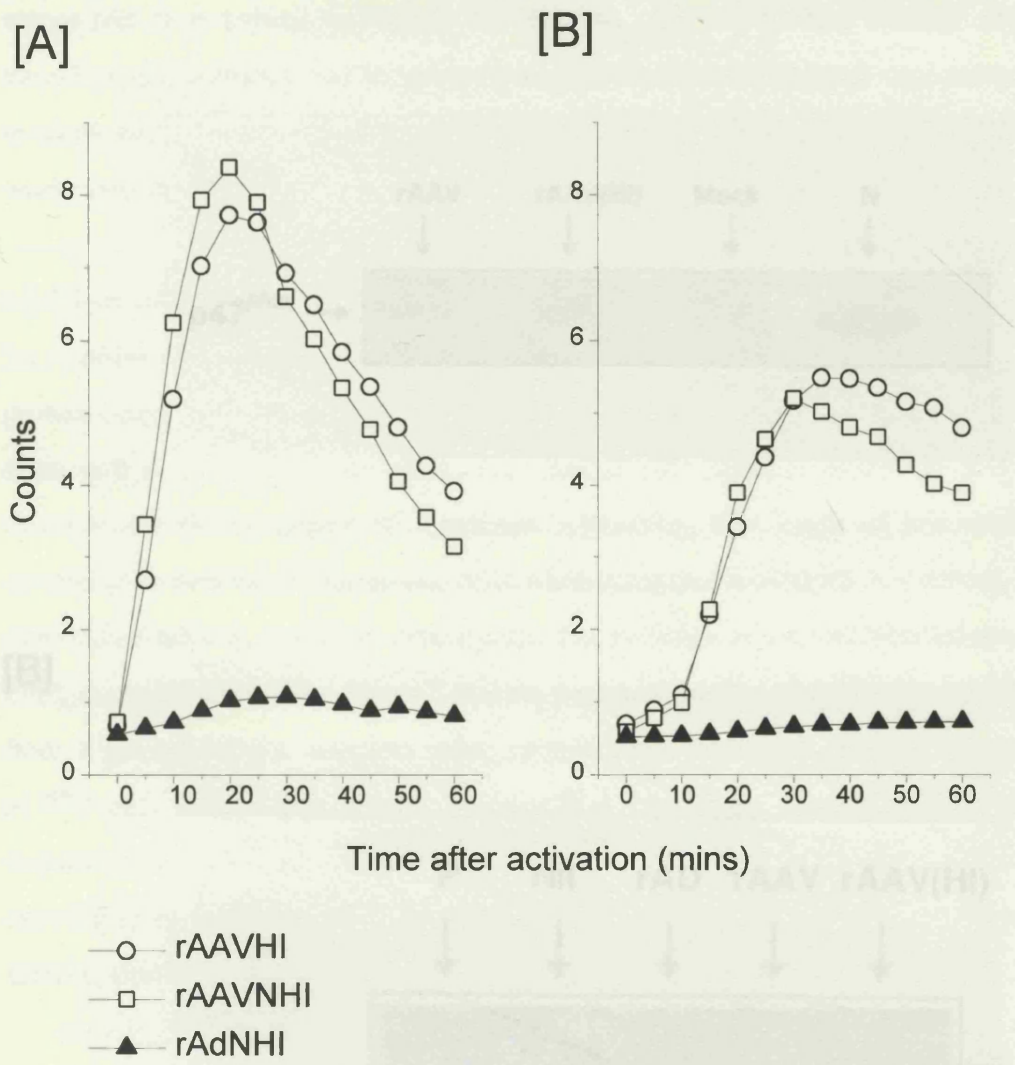
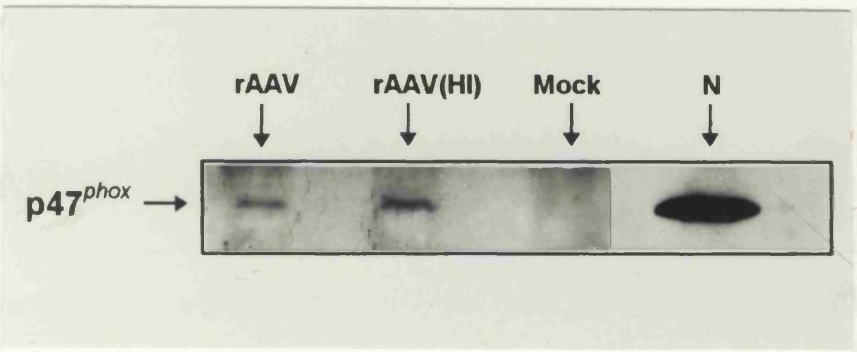


Fig 6.5. Restoration of function to immortalised p47^{phox}-deficient B cells by rAAV rescued from an adenoviral vector. 5.10^5 cells from one patient were incubated with rAAV particles recovered by infection of a producer 293 cell line with recombinant adenovirus p47AD2, and co-transfection of AAV replicative and encapsidation functions. rAAV supernatant was either heat-inactivated at 56^0C for 20 minutes to destroy the adenovirus (rAAVHI) or used directly for transduction (rAAVNHI). the same experiment was performed in parallel using adenovirus supernatant alone. At one month [A] and 2 months [B] post transduction, 2.10^6 viable cells were harvested and assayed for production of superoxide by chemiluminescence following stimulation with PMA. Significant restoration of function over time was observed in those cells exposed to rAAV, but not in those exposed to adenovirus alone.

[A]



[B]

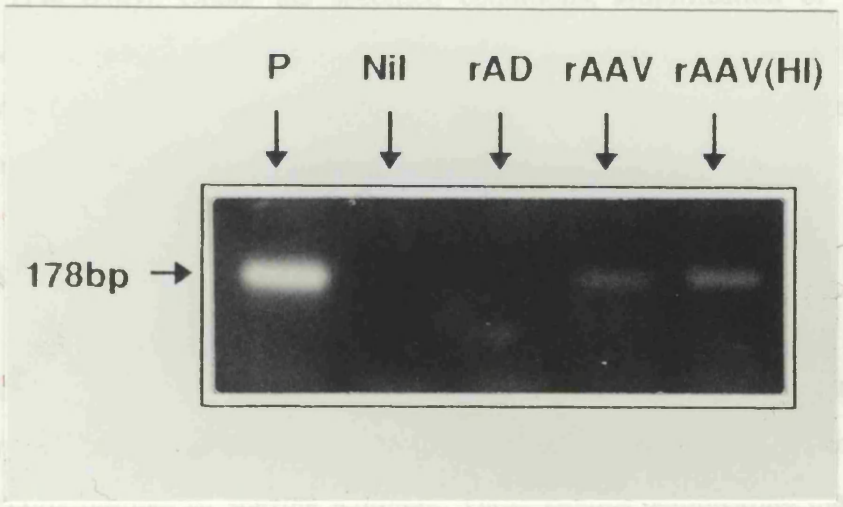


Fig 6.6. Identification of the vector genome in transduced cells, and expression of $p47^{phox}$ protein. [A] 8 weeks following infection with rAAV supernatant, protein extracts were separated by SDS-PAGE and after transfer to nitrocellulose, reacted with specific antiserum to $p47^{phox}$. Compared with a normal control (N), low levels of protein are expressed in cells exposed to rAAV supernatants, one of which was heated to 56^0C for 20 minutes to inactivate contaminating rAd (rAAVHI). Cells exposed to a mock supernatant (mock) failed to express protein. [B] The recombinant genome was detected by PCR specifically in cells infected with rAAV (rAAV and rAAV(HI)). Uninfected cells, or cells which had been infected with rAd supernatant alone failed to generate a signal. (P) is a positive control obtained by amplification from the rAd vector plasmid.

inactivate rAd, showed similar levels of activity. Furthermore, it was not possible to rescue rAd from culture medium or cell extracts after two months, whether or not the transducing supernatant had been heat treated. Cells exposed to mock supernatant failed to show activity above background obtained from untransduced cells at both 4 and 8 weeks post transduction.

6.3.5 Identification of the rAAV vector genome and protein expression

To confirm the presence of immunoreactive $p47^{phox}$ in transduced cell populations, protein extracts were obtained from transduced and mock transduced $p47^{phox}$ deficient B cells at 8 weeks post transduction, and reacted with specific antiserum (Fig 6.6A). Consistent with the degree of functional restoration, low levels of immunoreactive protein were detected in transduced cells when compared to normal. In contrast, protein was undetectable in mock transduced cells. The presence of the recombinant genome in DNA extracted from cell cultures 2 months post transduction was demonstrated by PCR from a genomic DNA template using exon primers that cross the first intron of the $p47^{phox}$ gene (Fig 6.6B). Under the specified conditions, amplification of intronic sequences does not occur. As predicted from the expression data, only those cultures exposed to rAAV supernatants, and which had shown some restoration of functional activity, produced a 178bp signal specific to the recombinant genome.

6.4 DISCUSSION

Enhanced delivery of the vector genome to cells in which rAAV is propagated may allow recovery of higher titres of rAAV, simply by increasing the number of productive cells and the copy number of starting template. These studies demonstrate that rAAV particles can be efficiently rescued and amplified from E1-deleted adenoviral vectors incorporating AAV vector sequences in the absence of wtAd. Furthermore, rAAV particles generated in this way successfully restore function to cells derived from patients with autosomal recessive CGD.

The failure to considerably improve on rAAV titres achieved by conventional methods is primarily due to the requirement for physical transfection of packaging plasmid

encoding *rep* and *cap*, but may be enhanced by optimisation of adenoviral delivery of the vector genome to producer cells, or perhaps by increased expression of adenoviral E1A functions. Generation of a parallel adenoviral vector expressing Rep and Cap (pXCJL.ΔBal) might overcome this limitation and would generate high copy numbers in each producer cell, but rescue of recombinant virus in 293 cells has proved elusive, presumably due to the complex effects of Rep expression on both cellular function, and replication of adenovirus. Strategies using inducible promoters to control expression of Rep are under investigation.

These studies further endorse rAAV as a promising vector system for somatic gene therapy of CGD and other haematopoietic disorders, but highlight the difficulties associated with production of useful titres of recombinant virus. Optimisation of AAV vector delivery to producer cells using adenoviral vectors, and development of packaging cell lines in which *rep* and *cap* are stably incorporated, should overcome some of the problems associated with propagation of recombinant virus, and may lead to recovery of higher titre rAAV. Furthermore, successful rescue of the AAV genome from an adenoviral vector may have useful direct application for transduction of target cells if Rep is transiently co-expressed. This strategy would utilise the high efficiency of gene transfer provided by the adenovirus to deliver the recombinogenic rAAV genome to the nucleus.

6.5 SUMMARY: RECOVERY OF rAAV FROM ADENOVIRAL VECTORS

- *rAAV particles can be efficiently rescued from E1-deleted adenoviral vectors by transient expression of AAV packaging functions in an E1-complementary permissive cell line.*

- *Levels of contamination with wild-type adenovirus are minimised.*

- *Direct recovery of the AAV genome from an adenoviral vector may be useful for stable transduction of target cells.*

CHAPTER 7:

MOLECULAR DIAGNOSIS BY GENETIC COMPLEMENTATION

7.1 INTRODUCTION

In a recent study, approximately two thirds of affected families were found to have the X-linked form of CGD, arising from an abnormality of the β-subunit of the flavocytochrome (Casimir et al. 1991). The remaining patients had autosomal recessive defects, with those exhibiting deficiency of $p47^{phox}$ the most prevalent. Defects in the α-subunit of the flavocytochrome, and in $p67^{phox}$ made up the remaining 10% of cases. This observed genetic distribution of CGD is in close agreement with a similar study of 82 American families (Clark et al. 1989). Molecular lesions at CYBB and CYBA, the genetic loci for $gp91^{phox}$ and $p22^{phox}$ respectively, are particularly heterogeneous, and unique to individual families in over 90% of cases described. In contrast, a GT dinucleotide deletion at a GTGT repeat at the boundary between the first intron and second exon of the gene accounts for over 90% of mutant alleles in $p47^{phox}$ deficient CGD (Casimir et al. 1991).

The advent of treatment by somatic gene therapy necessitates the identification of the defective component in all patients, and the demonstration that gene transfer restores function to cells derived from individual patients. Immunoblotting for components of the NADPH-oxidase identifies the defective protein in the majority of cases. However, molecular lesions in either component of flavocytochrome b_{558} usually results in absence of the entire heterodimer, and although the affected component may be inferred from inheritance, it remains difficult to distinguish between males with normal maternal NBT patterns, who may have acquired a new mutation at CYBB, and those with autosomal recessive deficiency of $p22^{phox}$. Identification of the defective component may also be difficult in rare individuals who express normal levels of functionally deficient protein. In these cases, complementation studies in a cell-free system using recombinant protein (Rotrosen et al. 1993), or in whole cells by monocyte fusion (Roos et al. 1992), may be helpful but can be technically difficult.

The inability of the adenoviral genome to transmit to progeny cells in the absence of continuing infection limits practical application as a vehicle for transduction of haematopoietic progenitor cells. However, in contrast to current retroviral gene delivery systems, adenoviral vectors efficiently transduce cells which are not actively in cell-cycle, and are therefore ideal for transduction of peripheral blood monocytes which undergo terminal maturation and differentiation in fixed tissues, but do not readily enter the cell-cycle. This application of adenovirus-mediated gene delivery provides a functional assay for molecular diagnosis of CGD by genetic complementation of primary monocytes, and may have therapeutic potential in the short term.

7.2 TRANSDUCTION OF MONOCYTES

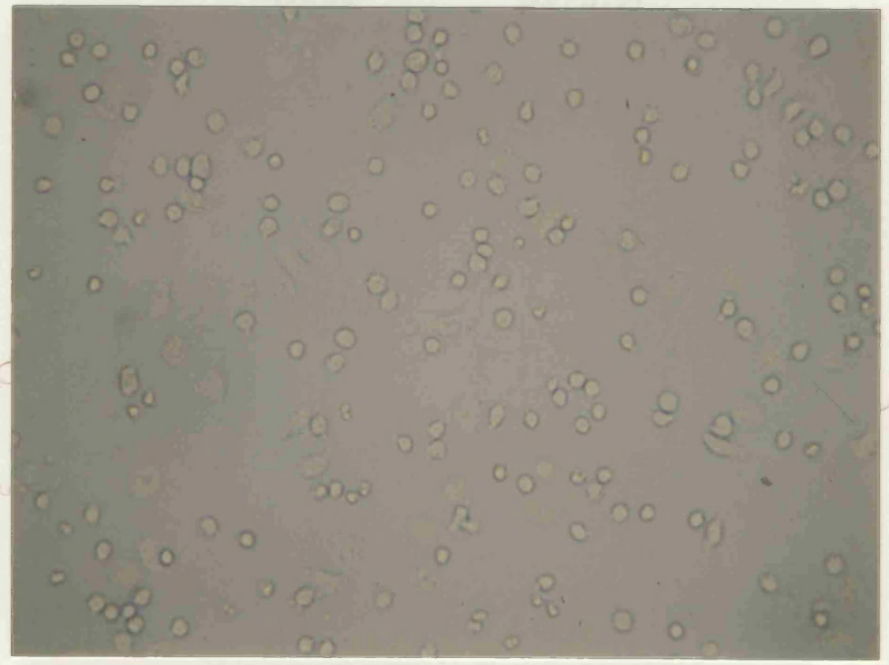
7.2.1 Transduction of CGD monocytes

Production of the adenoviral supernatant p47AD2, and the ability of this virus to complement the genetic defect in $p47^{phox}$-deficient B lymphoblastoid cells has been described previously. In contrast, transduction of similar cells derived from patients with known deficiencies of the flavocytochrome or $p67^{phox}$ failed to restore function (not shown). Peripheral blood monocytes prepared from three CGD patients were transduced with p47AD2 at a m.o.i. of 100-500. To control for non-specific effects arising from adenoviral infection, equal numbers of monocytes were subjected to sham infection with PBS alone, and to infection with a parallel adenoviral vector encoding LacZ (pLacZAD), at a similar m.o.i. Monocytes recovered from a normal individual were treated in the same way, and were used as a positive control.

7.2.2 Functional analysis

After 18-24 hours, monocytes were tested for their ability to reduce nitroblue tetrazolium (NBT) to insoluble and dark blue staining formazan (Fig 7.1, Fig 7.2).

[A]



[B]

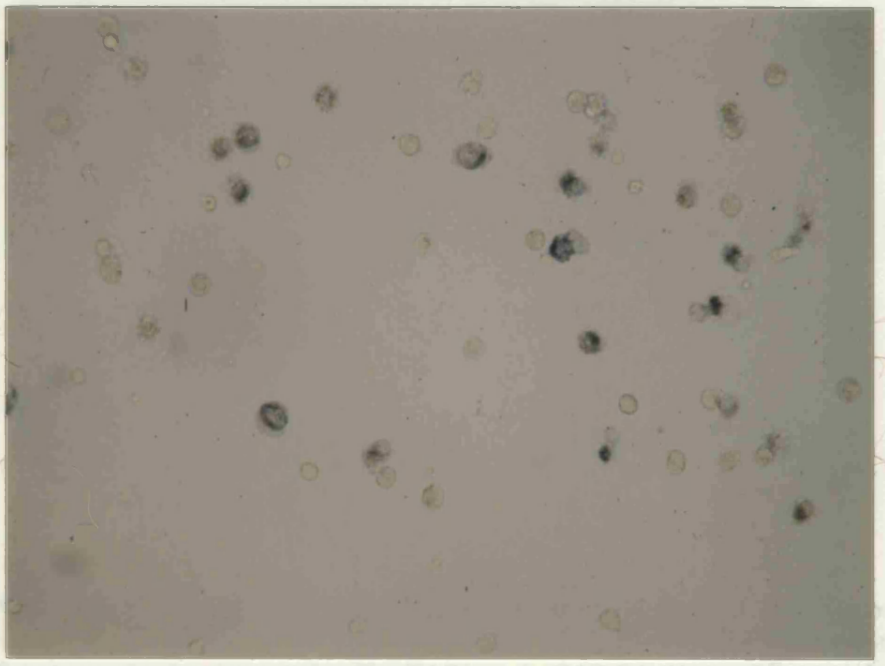


Fig 7.1 NBT staining of transduced monocytes. Adherent mononuclear cells purified from 5 mls of peripheral blood from one patient known to be $p47^{phox}$-deficient, were incubated separately with [A] PBS, or [B] p47AD2 recombinant adenoviral particles, for two hours. 18-24 hours post transduction, medium was removed and cells washed three times with PBS. NBT assay mixture was added directly to the adherent cells, and positive staining scored after 30 minutes incubation at 37^0C by light microscopy. A significant proportion of transduced cells show a positive NBT reaction, determined by precipitation of insoluble blue formazan onto the cells. In a control experiment, 100% of monocytes prepared from a normal individual were NBT positive.

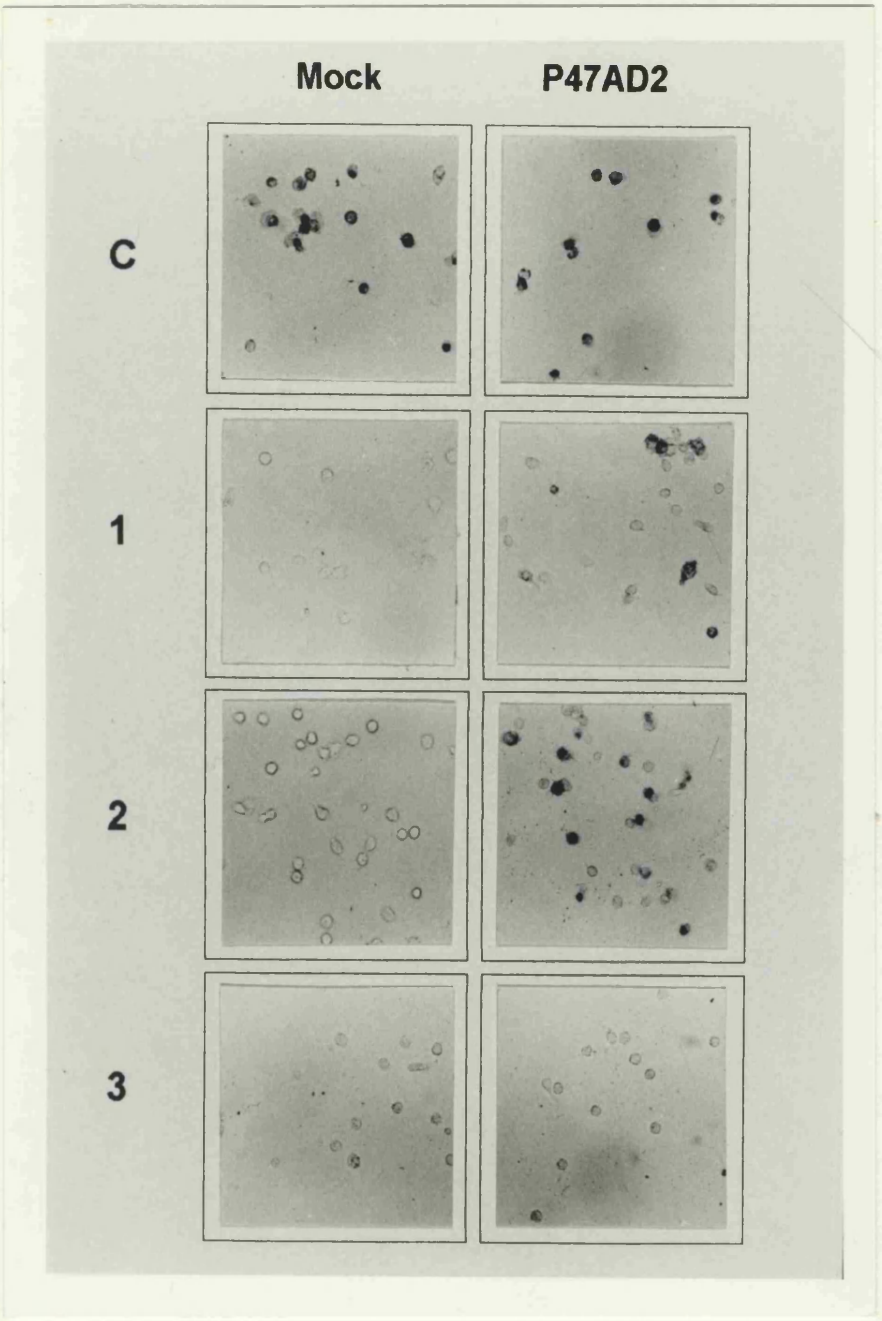


Fig 7.2 NBT staining of transduced monocytes. Adherent mononuclear cells purified from 5 mls of peripheral blood from three patients (1, 2 and 3) with CGD of whom patient 1 was known to be $p47^{phox}$-deficient, were incubated separately with p47AD2 or pLacZAD (Mock) recombinant adenoviral particles for two hours. 18-24 hours post transduction, medium was removed and cells washed three times with PBS. NBT assay mixture was added directly to the adherent cells, and positive staining scored after 30 minutes incubation at 37^0C by light microscopy. Cells from patient 1 and patient 2, transduced by p47AD2, both show positive staining in a proportion of cells. In contrast, cells from patient 3 under all transduction conditions remained negative. All CGD cells transduced by the LacZ encoding adenovirus (Mock) remained NBT negative. Control cells (C) were derived from a normal individual.

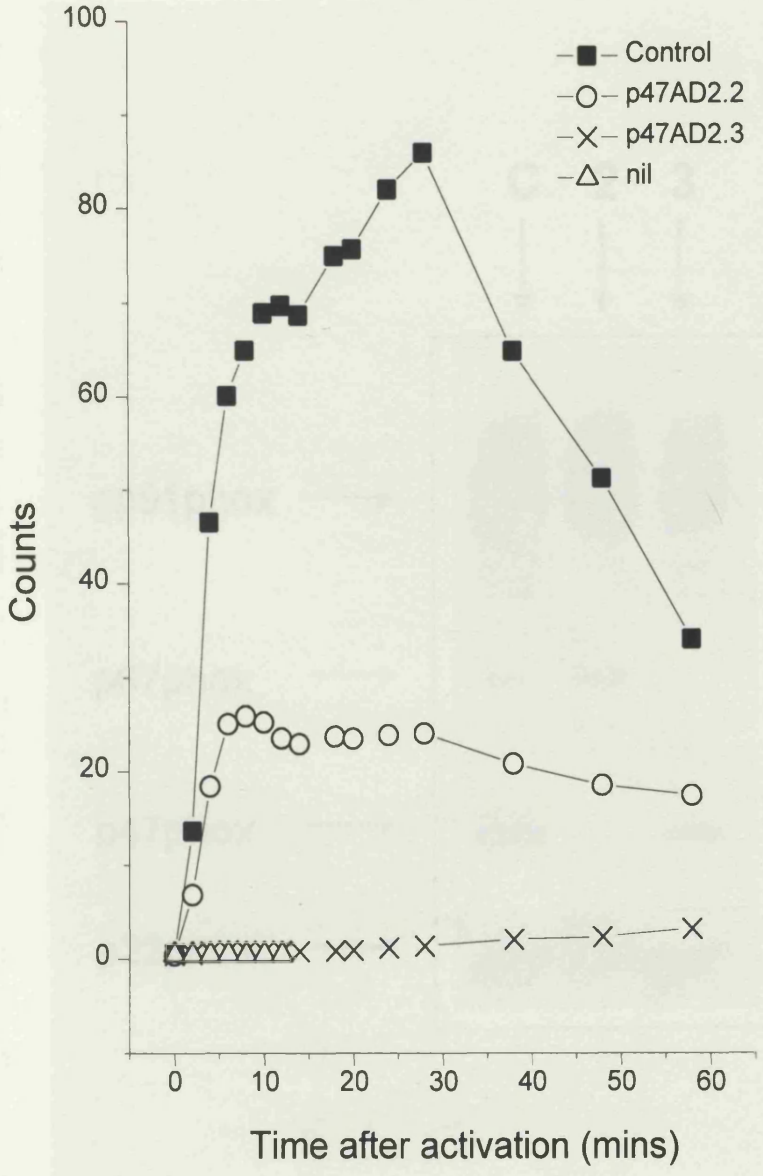


Fig 7.3 Functional reconstitution of monocytes. Adherent mononuclear cells purified from 5 mls of peripheral blood from two patients (patients 2 and 3) with CGD of undetermined molecular pathology, were incubated separately with PBS alone, p47AD2 or pLacZAD recombinant adenoviral particles for two hours. 18-24 hours post transduction, cells were harvested by scraping and assayed by chemiluminescence for production of superoxide following stimulation with PMA. Only cells from patient 2 transduced by adenovirus encoding $p47^{phox}$ (p47AD2.2) showed significant restoration of function. Similar cells which underwent sham transduction with PBS, or transduction by adenovirus encoding LacZ remained inactive (not shown). No activity was detected in cells obtained from patient 3 under all conditions, (p47AD2.3). Control and nil represent unmanipulated cells derived from a normal individual and a patient respectively.

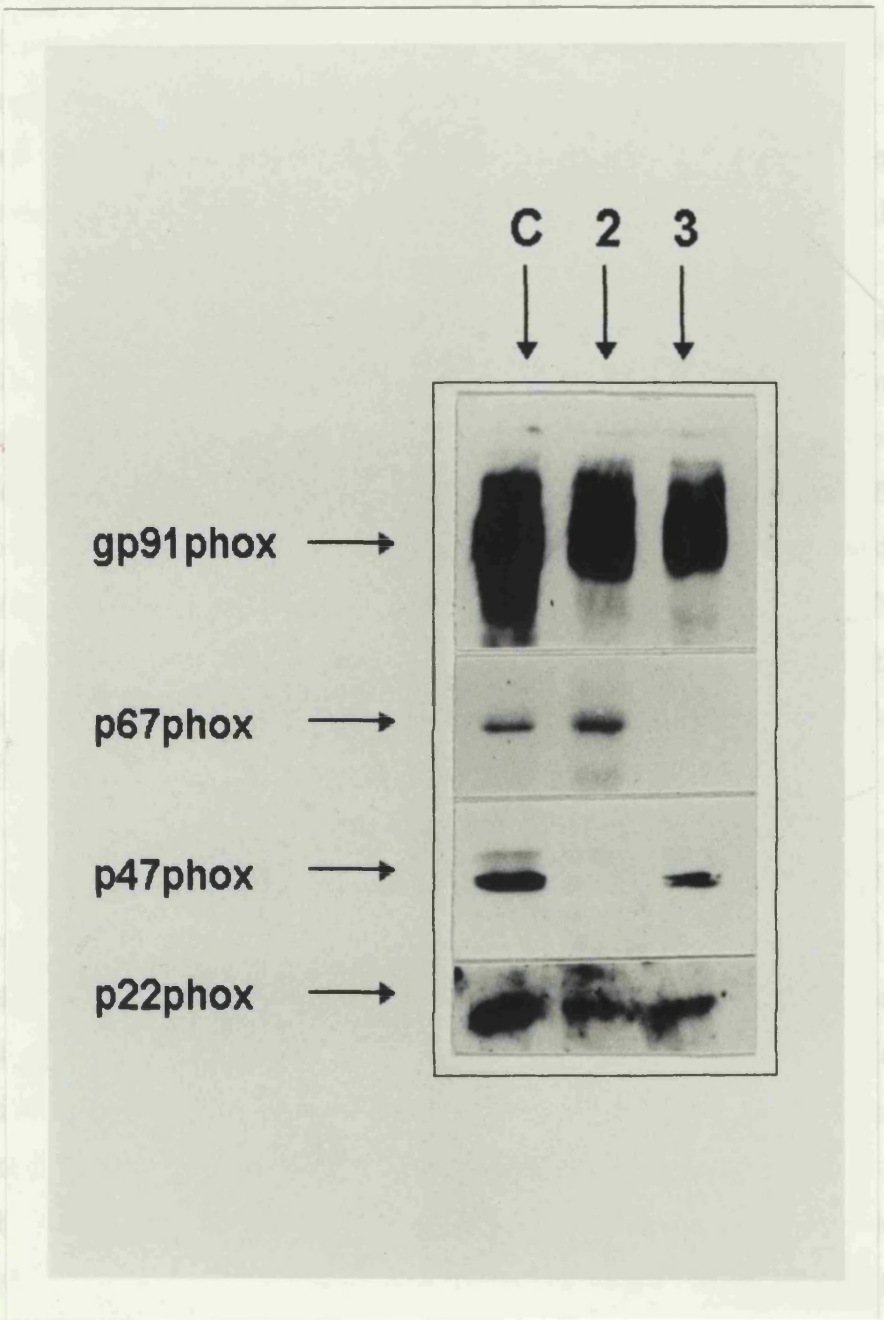


Fig 7.4 Western Blot analysis of molecular lesions. Crude protein extracts prepared from neutrophils of patients 2 and 3 and separated by PAGE, were electroblotted to nitro-cellulose, and reacted with specific antisera for each component of the NADPH-oxidase. As predicted from the functional data, patient 2 lacked $p47^{phox}$. In contrast, patient 3 expressed normal levels of immunoreactive $p47^{phox}$, but was deficient in the other cytosolic component $p67^{phox}$. Control extracts (C) are derived from a normal individual.

Patient 1, a boy, was known to be $p47^{phox}$ deficient from previous studies (Casimir et al. 1992). Patients 2 and 3, both girls, were newly diagnosed by NBT staining of peripheral blood neutrophils, but the site of the molecular lesion was not known. Following transduction by p47AD2, a proportion of monocytes derived from both patients 1 and 2 attained the ability to reduce NBT, whereas monocytes from patient 3 remained negative. Control cells from a normal individual stained 100% positive. All CGD monocytes that had undergone sham infection, or which were infected with a LacZ encoding adenovirus remained 100% NBT negative, indicating that the correction was specific. Further evaluation of NADPH-oxidase function was performed by chemiluminescent assay for stimulated generation of hydrogen peroxide in monocytes derived from patients 2 and 3 (Fig 7.3). Confirming the findings of the NBT test, monocytes from patient 2 exhibited significant return of activity when compared with a normal individual, which again was specific to those cells exposed to p47AD2. Patient 3 failed to demonstrate activity under all conditions, suggesting as before that the detrimental molecular lesion affected an alternative component of the NADPH-oxidase to $p47^{phox}$.

7.2.3 Assignment of defective component by Western blot analysis

To confirm the specificity of the responses to infection by an adenoviral vector encoding a functional copy of $p47^{phox}$, crude protein extracts obtained from neutrophils of patients 2 and 3 were subjected to Western blot analysis (Fig 7.4). As predicted from functional responses, patient 2 was found to lack immunoreactive $p47^{phox}$. In contrast, patient 3 expressed normal levels of $p47^{phox}$, but lacked the cytosolic factor $p67^{phox}$, which accounts for the inability of p47AD2 to restore functional activity to these monocytes. Patient 1 has previously been shown to lack $p47^{phox}$, and all three patients expressed normal levels of flavocytochrome.

7.3 DISCUSSION

Gene transfer to lymphoblastoid cell lines and to haematopoietic progenitors derived from peripheral blood of patients with CGD, has been reported by several groups (Thrasher et al. 1992; Porter et al. 1993,1994; Chanock et al. 1992; Volpp and Lin. 1993; Sekhsaria et al. 1993; Lei et al. 1994). Efficient transduction of primary CGD

monocytes with components of the NADPH-oxidase has not previously been described, in part due to the inefficiency of retrovirus mediated gene delivery to quiescent cells. In contrast to retrovirus-based gene delivery systems, adenoviral vectors efficiently transduce non-dividing cells, but are unable to produce stable correction of progeny, and are therefore of limited use as agents for curative therapy of haematopoietic disorders. However, successful therapeutic use of allogeneic leukocyte transfusions for recalcitrant or life threatening infections in CGD raises the possibility that genetically modified autologous monocytes may be a target for transitory gene therapy (Buescher and Gallin, 1982). Animal models generated by gene targeting will help answer this question (Pollock et al. 1995).

The prospect of curative somatic gene therapy for haematopoietic disorders by transduction of pluripotent haematopoietic stem cells, necessitates that the molecular lesion for all candidate patients is defined, and that expression of a normal copy of the affected gene results in restoration of function. Genetic classification of a significant proportion of flavocytochrome deficient male patients, who have acquired a mutation at CYBB from the maternal germline, and who have normal maternal NBT patterns, and other patients who express normal levels of immunoreactive but non-functional protein, remains problematical. Complementation in the cell-free system with recombinant protein, or complementation within monocyte heterokaryons may be useful in these cases, but these procedures are time-consuming and can be technically difficult.

Transduction of primary monocytes by adenoviral vectors permits reliable identification of defects within individual components of the NADPH-oxidase, and confirms restoration of functional activity by gene transfer. The assay is specific, is simple to perform, requiring little patient sample preparation, and does not require large numbers of cells. Construction of additional vectors encoding each component would allow rapid molecular diagnosis of all known forms of CGD by genetic complementation.

7.4 SUMMARY: GENETIC COMPLEMENTATION OF CGD MONOCYTES

- *Adenoviral vectors encoding oxidase components complement the genetic defect in primary monocytes derived from CGD patients.*

- *Genetic complementation results in functional reconstitution of the NADPH-oxidase.*

- *Adenovirus-mediated complementation offers a rapid means for molecular diagnosis, and may provide a short-term therapeutic strategy.*

CHAPTER 8:

DISCUSSION

8.1 GENE THERAPY FOR CGD

As a monogenic recessive disorder with well defined molecular characteristics, CGD is an ideal candidate for therapeutic intervention by gene transfer. For this strategy to be successful, the efficiency and stability of gene transfer must be high, and expression levels sufficient to sustain correction of both the biochemical and clinical phenotype.

8.1.1 Target cell populations

The optimal target cell population for gene transfer is the pluripotent haematopoietic stem cell (PHSC), which is defined by the capacity for extensive self-renewal, and retention of multilineage differentiation potential (Keller, 1992). In the absence of a specific marker or assay system for self-renewal, the term stem cell refers to a heterogeneous and hierarchical population of cells that are capable of long-term reconstitution of the haematopoietic system of recipient animals.

8.1.2 Self-renewal and differentiation of stem cells

Exposure of murine bone marrow to anti-proliferative agents has little effect on the number of multipotential progenitors, and fails to inhibit formation of late-appearing spleen colonies (representing cells with some long term repopulating ability) or blast cell colonies (Ogawa, 1993). This is indicative of the fact that in the steady state, the majority of stem cells are dormant in the cell cycle (G_0) and only a few cells supply all haematopoietic cells at a given time. Studies in mice using retroviral vectors to label individual stem cell clones provide the best evidence for clonal succession (Kay, 1965), and also provide indirect evidence for stem cell quiescence. This period of dormancy may be necessary for maintenance of genetic integrity, and may safeguard against exhaustion of the stem cell pool during periods where rapid cell production is required.

The decision of a stem cell to self-renew or differentiate is a stochastic process (Till et al. 1964). Individual blast cell colonies, replated *in vitro*, generate heterogeneous

distributions of secondary blast colonies (representing self-renewal), or multilineage colonies (representing differentiation) (Nakahata and Ogawa, 1982). Cytological analysis of multilineage murine colonies derived from a single cell origin suggest that pluripotential stem cell commitment (selection of lineage potential during differentiation) is also a stochastic process (Suda et al. 1983), and is supported by similar studies on isolated human progenitors (Leary et al. 1985). Alternatively, lineage commitment may be cytokine regulated, or stochastic at the level of the stromal environment (Metcalf, 1991). Whether PHSC are randomly committed to a single lineage, or give rise to oligopotential progenitors is unknown.

Survival and expansion of progenitor cells is directed by an increasing number of interacting cytokines (Table 8.1) (Moore, 1991; Ogawa, 1993). These operate at 3 different levels, and have become important components of *ex vivo* gene transfer protocols in which survival and proliferation of target bone marrow cells must be optimised for successful transduction. However, advantages to the efficiency of progenitor cell transduction may be traded for detrimental effects on PHSCs, which may under certain conditions lose self-renewing ability, and become terminally differentiated. The most frequently used assay systems do not address this question.

Table 8.1 Cytokine action on haematopoietic progenitor cells

Level of cytokine action	*Function*
Late-acting lineage-specific	Proliferation and maturation of committed progenitors
Intermediate-acting	Proliferation of multipotential progenitors after exit from G_0 and synergy with lineage specific factors
Early-acting lineage non-specific	Control kinetics of cell-cycle dormant primitive progenitors

8.1.3 Evaluation of PHSC progenitor cell transduction

In lethally irradiated murine models, 30-40% of cells participating in long term engraftment of the animal can be routinely and stably transduced *ex vivo* by the current generation of retroviral vectors. However, transfer of this technology to humans, non-human primates or other large out-bred animals has been much less successful (Van Beusechem et al. 1990, 1992; Brenner et al. 1993a, 1993b; Bodine et al. 1993). Typically less than 0.1% of peripheral blood cells are marked long term by retroviral vectors encoding reporter genes. The reasons for this discrepancy are uncertain, but probably reflect poor understanding of culture conditions required to maintain PHSCs, failure to induce cell-division without differentiation in this population, or deficiency of cell-surface amphotropic receptors.

One major problem is the lack of an experimental assay system for the PHSC population. In contrast, transduction of later progenitors can be determined *in vitro* by growth factor-dependent surrogate assay, based on clonal growth of single cells seeded in semi-solid medium (Watt and Visser, 1992). Primitive haematopoietic progenitors can be quantitated in long-term marrow cultures supported on stromal layers, by detection of clonogenic cells over a five to eight week period in the long term culture initiating cell assay (LTC-IC) (Sutherland et al. 1989). However, these cells represent a heterogeneous population, and are clearly not necessarily representative of the PHSC pool. This can be deduced from retroviral marking studies in which high efficiency gene transfer to cells detected by 8 week LTC-IC, fails to correlate with long term engraftment.

A more informative assay, and one which tests repopulating ability, is based on transplantation of human haematopoietic progenitors into immune-deficient severe combined immunodeficient SCID or bg/nu/xid mice, creating chimaeric SCID-human (SCID-hu) haematopoiesis (McCune et al. 1988; Kamel-Reid and Dick, 1988; Dick et al. 1991). Studies using a SCID-repopulation model strongly suggest that very primitive cells can engraft the murine microenvironment, which in the presence of human cytokines can proliferate and differentiate into multiple myeloid, erythroid and B cell lineages (Lapidot et al. 1992). The exact relationship between the SCID-repopulating

cell (SRC), and the human stem cell is not yet known. However, repopulation of SCID and more recently of SCID/NOD mice (created by backcrossing the *scid* mutation onto the NOD/Lt diabetic strain background) which in addition to T and B cell deficiency, have defects of NK, macrophage and complement function, indicate that efficiency of retrovirus-mediated gene transfer to human SRCs is extremely low, and that the transduction procedure greatly diminishes the number of viable SRCs (Dick, 1995; Shultz et al. 1995). These models of human haematopoiesis therefore provide a powerful method for determination of gene transfer to very primitive cells, which in contrast to the LTC-IC, are probably significantly more representative of true PHSCs.

8.2 ENHANCED GENE TRANSFER TO PHSCs

The major difficulty with current retrovirus-based vector systems is an inability to transduce cells which are not actively in cell-cycle (Roe at al. 1993). This problem can be addressed in two ways. Culture conditions could be modified to induce stem cell-cycling, or to broaden the window for retroviral transduction, but in such a way that cell viability or repopulating and self-renewing potential are not compromised. Enrichment, purification, and *ex vivo* expansion of the PHSC pool would greatly facilitate this process. Alternatively, gene delivery systems which are not dependent on cycling status of the cells could be developed.

8.2.1 Identification of self renewing PHSC by surface phenotype

For bone marrow transplantation following chemotherapy, short-term repopulating donor cells may be sufficient to reconstitute multilineage haematopoiesis prior to regeneration of host bone marrow. However, for curative therapy of inherited disease, genetically manipulated 'donor' PHSC must contribute at least partially to permanent repopulation of the haematopoietic system. Although there is no direct assay for true self-renewing activity, successful long term reconstitution of SCID mice, lethally irradiated non-human primates, and curative BMT for genetic disease suggests that within the transplanted cell population, such activity must exist.

Fractionation of murine bone marrow suggests that the most primitive progenitors differ in both their physical and molecular characteristics from later progenitors that possess short-term repopulating potential. As few as 30 Thy-1 and Sca-1 antigen positive, and lineage marker negative (lin^-) murine marrow cells are able to rescue otherwise lethally irradiated mice (Sprangrude et al. 1988). Enrichment for human progenitors can be achieved by positive or negative selection with monoclonal antibodies or lectins directed against cell surface molecules, and on the basis of physical properties such as cell density, surface charge, light scatter, and cell cycle status (lin) (Visser, 1992). The most frequently used antibody for isolation of human haematopoietic progenitors is directed against CD34, a heavily glycosylated surface antigen which is preferentially expressed on haematopoietic progenitor cells (Simmons et al. 1992). It has also been detected on osteoclasts, bone marrow stromal precursor cells, peripheral nerve sheaths, and as a ligand for L-selectin on vascular endothelial cells (Holyoake and Alcorn, 1994). The pattern of glycosylation segregates with function, but is not yet determined for haematopoietic progenitor cells. CD34 antigen is expressed on 1-3% of low density bone marrow mononuclear cells, and about 0.1% of peripheral blood mononuclear cells. The $CD34^+$ population includes oligopotent and pluripotent progenitors and can reconstitute haematopoiesis in lethally irradiated non-human primates (Berenson et al. 1988), at least in the short term, and all haematopoietic lineages in SCID mice. These studies have now been extended to human transplantation following chemotherapy for malignancy (Berenson et al. 1991).

Multidimensional flow cytometric analysis permits characterisation of the $CD34^+$ population on the basis of surface immunophenotype, and can be used to enrich further for primitive progenitors. In particular, co-expression of the antigen Thy-1 on 5-10% of lineage negative, human $CD34^+$ bone marrow cells selects for a population with the potential for multilineage reconstitution of SCID-hu mice (Murray et al. 1995). Thy-1 negative populations in contrast produce little or no engraftment in SCID-hu models of human haematopoiesis. Further enrichment can be defined by a population which is $CD34^{hi}$, Thy-1^+, lin^-, and which expresses low or undetectable levels of HLA-DR (HLA-DR^-), stains dimly with the dye Rhodamine 123 (Rho^{dull}), and which expresses the RO isoform of the CD45 antigen ($CD45RO^+$). The primitive nature of these cells is

indicated by long term culture-initiating potential, formation of primitive blast colonies in culture, the ability of these colonies to form new blast colonies when replated, and multilineage engraftment of foetal liver, bone and thymus in a SCID-hu mouse model (Holyoake and Alcorn, 1994).

Although it is not yet possible to isolate a pure population of self-renewing pluripotent stem cells, enrichment of progenitor populations for these most primitive cells may facilitate *ex vivo* genetic manipulation. Recently, haematopoietic cells enriched for a population characteristic of very primitive progenitors (representing 1 in 10^5 bone marrow mononuclear cells), have been isolated by selection for cell-cycle quiescence in the presence of a metabolic poison (Berardi et al. 1995). Ultimately, it may be possible to expand this population by addition of relevant cytokines, and to retain both repopulating, and self-renewing capability (Williams, 1993).

8.2.2 Retroviral vector systems for transduction of PHSCs

Permissiveness of the host cell to transduction by retroviral vectors based on the murine leukaemia viruses is dependent on disruption of the nuclear membrane during mitosis at which time the pre-integration complex localises to the nucleus. In contrast, establishment of an integrated HIV-1 provirus is independent of cell cycle, because nuclear localisation of the pre-integration complex is mediated by active import. HIV-1 *gag* matrix protein is at least partially responsible for this property by virtue of a nuclear localisation sequence at the N-terminus. Incorporation of this sequence into homologous regions of MLV is an attractive possibility, but has not been reported. An alternative or additive explanation for poor transduction efficiency of PHSCs is determined by the frequency of cell-surface receptors. For reasons outlined previously, it has not been possible to determine the frequency of the amphotropic receptor (Ram-1) on human stem cells, but in whole marrow at least, it is expressed at lower levels than the homologous Gibbon ape leukaemia virus receptor (Glvr-1) (Kavanaugh et al. 1994). Retroviral vectors pseudotyped with the corresponding envelope may therefore mediate improved transduction efficiency of this cell population (von Kalle et al. 1994). Cell-surface expression of both receptors can probably be enhanced by cell culture in phosphate-depleted medium, and reflects their function as Na^+-dependent phosphate

symporters (Kavanaugh et al. 1994). Ultimately, by utilising stem cell-specific natural ligand-receptor interactions, viruses with chimaeric envelopes may be able to direct targeted transduction.

The titre of virus is probably less important for *ex vivo* manipulation of haematopoietic cells than for direct *in vivo* application. This is supported by several studies which have shown that the efficiency of progenitor infection in unseparated mononuclear marrow fractions, or purified $CD34^+$ cells is very similar, indicating that viral titre is probably not limiting (Van Beusechem et al. 1993; Correll and Karlsson, 1994). Furthermore, a combination of physical concentrating methods, and growth of producer cells to high cell numbers in bioreactor systems at 32^0C, can routinely achieve titres of over 10^7 infectious particles per ml of supernatant. For clinical application, this can be measured against a total of approximately 2.10^6 CD34+ cells per kg, which are necessary to reconstitute an ablated human haematopoietic system, at least in the short term.

8.2.3 Alternative virus-based vector systems

Adeno-associated virus is less dependent on cell cycle for transduction, and may therefore be useful for stem cell gene transfer. Transduction efficiency of cultured primary human fibroblasts in S-phase is about 200 times that of resting cells, but the vector genome persists for at least 12 days in culture, and can be recruited for transduction at any time (Russell et al. 1994). Furthermore, exposure to DNA damaging agents such as γ-irradiation, UV irradiation, tritiated thymidine, and cis-platinum increases transduction efficiency, particularly of stationary cells, by up to 750-fold (Alexander et al. 1994). Cytotoxic agents which do not directly damage DNA are ineffective, which possibly implicates cellular DNA repair mechanisms in the integrative process. Although the mechanism is unclear, there appears to be an increase in the number of input vector rAAV genomes converted to a form that allows gene expression. Whether this reflects integration, or expression from episomal templates is unknown. However, correlation between virus titres determined by growth of drug-resistant colonies, and staining for transduced cells, would suggest that transduction and integration are closely related events.

The ability of rAAV vectors to transduce PHSCs has not been addressed, partly because inefficient methods for production have restricted experimental application. However, successful transduction of $CD34^+$ progenitor cells has been reported for murine cells, human umbilical cord blood, and cells derived from patients with Fanconi anaemia and sickle-cell disease (Zhou et al.1993, 1994; Walsh et al. 1994; Miller et al. 1994). In this latter study, a multiplicity of infection of 500-1000 rAAV particles produced transduction efficiencies of between 20 and 40%, determined by clonogenic assay in semi-solid medium, and PCR-based detection of vector-specific transcripts in individual colonies.

Production of high titre stocks of rAAV particles is at present difficult, but may be enhanced by optimisation of component transfer to producer cells, for example using recombinant adenoviral vectors. Alternatively, Rep and Cap functions could be stably incorporated in a packaging cell line, similar in principle to those used to generate recombinant retroviruses. This has been problematical predominantly because of the cytostatic effect of Rep, which also explains the difficulty in recovering adenoviral vectors encoding this protein. Incorporation of the highly specific regulatory elements of the *E.coli* tetracycline resistance operon, for example, may overcome these problems.

Several cell lines have been constructed in which Rep is expressed from inducible promoters, but the ability of these cells to produce higher titre virus in the absence of wild-type AAV is not yet known (Holscher et al. 1994: Yang et al. 1994). Utilising the fact that HeLa cells express YY1 in the absence of E1A, a polyclonal HeLa cell line was constructed in which packaging functions are stably expressed from the natural AAV promoters, and which should therefore induce at the time of adenoviral infection (Thrasher et al. unpublished). These cells are relatively resistant to transfection by physical means, but even when infected with an adenoviral vector incorporating rAAV vector genome sequences, fail to produce significant amounts of single-stranded progeny DNA. This property has recently been described by another group, who also showed that co-transfection of expression vectors encoding all forms of *rep* reconstituted production of ssDNA. The mechanisms underlying these observations are unclear, but may reflect a relative deficiency of capsid protein in the absence of

sufficient *trans*-activating Rep, and which is known to be necessary for efficient accumulation of ssDNA species (Hermonat et al. 1984; Tratschin et al. 1984). Stable introduction of vector sequences into the same HeLa cell line resulted in low grade production of virus in the absence of helper, but levels did not markedly increase co-incident with adenovirus super-infection. Increasing the copy-number of the packaging genome may overcome the restriction on virus production, and would simulate events of a natural lytic wtAAV infection in which multiple transcriptional templates are generated. Unfortunately, introduction of sufficient heterologous DNA into a wtAAV genome to exceed the size limit for packaging leads to replication, but also to high levels of wild-type contamination arising from internal deletion or recombination with vector sequences. Alternatively, packaging functions with deleted TR sequences could be incorporated in an adenoviral vector as described, or in a plasmid containing the SV40 origin of replication, which would be replicated to high copy number in the presence of SV40 large T-antigen (TAg). Inducible expression or temperature-dependent activity of TAg, might allow construction of stable packaging cell lines based on these properties.

Generation of wtAAV is undesirable for gene therapy applications, and considerably reduces the efficiency of packaging of the recombinant genome. Although it is possible to design a TR-deleted packaging construct, and a vector construct with no overlapping sequences, the proposed model for wtAAV integration whereby Rep binds to TR sequences and similar sequences at the integration locus, suggests that complexes could form between the TR and p5 promoter Rep-binding site (Kotin, 1994). Separation of Rep and Cap functions on different plasmids, and use of heterologous promoter or polyadenylation sequences should significantly reduce the chance of wild-type production.

Conventional adenoviral vectors are limited in their application to haematopoietic disease because of instability of transduction. However, hybrid Ad/AAV vectors, in addition to their usefulness for generation of rAAV in the absence of wtAd, may overcome some of the problems imposed by each system alone. In particular, transient or inducible expression of Rep *in trans* may mediate excision of the AAV vector

genome from the adenoviral vector, and targeted integration on chromosome 19. Expression of *rep* from outside the AAV TR sequences would ensure maintenance as an episome, and subsequent loss with cell division. This system derives the benefits of efficient transduction of non-dividing cells by adenoviruses, and the site-preferential integrative mechanisms of rAAV. Expression of heterologous genes from this locus has not been examined in detail, but may have to counteract mechanisms utilised by wtAAV to establish latent infection. A similar principle could be employed for plasmid vectors, and although less efficient, would not be compromised by expression of toxic and immunogenic Ad proteins in the host cell, which may themselves critically alter the cellular phenotype.

8.3 SAFETY ASPECTS

Safety of new therapeutic procedures must be weighed carefully against conventional therapies and against the likely benefits to be gained by the patient. This is particularly true of gene transfer. As a potentially curative procedure for an immunodeficiency such as CGD, gene transfer and re-engraftment of autologous stem cells can be measured against bone marrow transplantation, which from a fully matched sibling donor is probably associated with a 20-30% average chance of mortality directly related to the degree of immunosuppression required for engraftment (Morgan, 1992). The necessity for immuno-suppressive conditioning prior to engraftment of genetically modified cells is partially dependent on the efficiency of gene transfer, but would significantly add to the risks associated with the procedure. Several animal studies have now shown that long-term chimaerism can be established without bone marrow ablation, and would imply that efficiency of gene transfer is the limiting factor (Stewart et al. 1993; Wu and Keating, 1993; Bienzle et al. 1994).

8.3.1 Safety of gene delivery systems

The safety of gene transfer relates to the effects of expression of the inserted gene on the host cell, and to unrelated effects arising from expression of vector proteins, or from disruption of normal cellular function. The first of these is gene-specific, and may require regulated differentiation and tissue-specific expression. This is less of a problem

for haematopoietic cells which can be isolated and manipulated *ex vivo*, and to a certain extent obviates the need for specific cell-targeting strategies. Expression of vector protein in target cells has become a particular problem for adenovirus-based delivery systems, and results in toxicity at high copy number, and induction of powerful immune responses. This is not a problem for gene transfer by retroviral or AAV-based vectors which do not encode any viral proteins.

Production of recombinant virus free from contamination by replication-competent wild-type or helper virus is of paramount importance for clinical application. Three monkeys infected with a recombinant retroviral supernatant heavily contaminated with helper virus developed high grade T cell lymphomas from which helper virus sequences were recovered (Donahue et al. 1992). The frequency of helper contamination can be minimised by construction of more sophisticated cell lines in which homology between vector and helper sequences are minimised, and in which multiple recombination events are necessary for production of replication competent virus. In the absence of helper contamination, the risks associated with these vectors are associated with disruption of normal cellular function secondary to integration into the host cell genome. Site-preferential integration mediated by AAV Rep proteins may be advantageous, but is not currently applicable, and may not support high level expression of heterologous genetic material.

For essentially random integration events, insertional mutagenesis occurs when a vector integration event leads to disruption or abnormal regulation of a gene. The chance of insertion and inactivation of both copies of a gene involved in regulation of cell-cycle, for example a tumour suppresser gene has been estimated to be 1 in 10^{12}. Tumour formation by non-acute transforming viruses, including the murine leukaemia viruses is thought to arise by recombination with endogenous viruses, and activation of cellular genes secondary to proviral integration events. Transformation probably involves activation of multiple proto-oncogenes by promoter insertion (Cloyd et al. 1980; Cuypers et al. 1984). Mitogenic stimuli transduced by binding of envelope protein to cell-surface receptors may also be important (Li and Baltimore, 1991). While transformation secondary to integration of the recombinant provirus presents a finite

risk in primate cells, little homology is shared between human endogenous retroviral sequences (HERV) and MLV-based vectors, so that activation of a replication competent virus by this mechanism is unlikely.

Transfer of defective endogenous murine retroviral sequences originating from the packaging cell line, the most abundant of which are the virus-like 30S RNAs (VL-30s), and intracisternal A-type particles (IAP), has been reported to occur at low levels, but has not been associated with pathogenicity (Hatzoglou et al. 1990). VL30 sequences can be packaged into type C virions, and horizontally transmitted to other cells. However, protein products of these RNAs have not been reported. Proviruses of murine IAPs are particularly abundant, and are expressed in mature B cells, B cell-related tumours, and embryos (Kuff and Lueders, 1988). They most probably represent retrotransposons recently descended from retroviruses, and are not known to encode infectious virus. For AAV-based vectors, risks of promoter insertion are again finite, and partially determined by the nature of the promoter used to direct expression of the transferred gene. Similarly, replacement of retroviral LTR regulatory sequences in the U3 region by natural sequences may restrict the risk of a detrimental integration event to cells in which the promoter is active.

8.3.2 Non-viral methods for gene transfer

Non-viral methods for gene transfer were first developed in the early 1970's, and have distinct safety advantage over virus-based systems because no viral genetic material is co-transferred. They include chemical techniques, mechanical techniques, membrane fusion and receptor mediated endocytosis. The most commonly used laboratory methods for transfer of DNA to recipient cells are based on co-precipitation and endocytosis of DNA with calcium phosphate, and in resistant cases, electroporation. However, the efficiency of stable gene transfer is generally very low, and these techniques are not useful in a clinical setting.

Two techniques that may have clinical application are based on liposome-mediated membrane fusion (Alton et al. 1993), and on receptor-mediated endocytosis of conjugated DNA (Michael and Curiel, 1994). The main disadvantages of liposome-

mediated gene transfer are low efficiency, and low frequency of stable long term expression. To overcome some of these problems, molecular conjugate vectors have been developed in which DNA is delivered via a receptor-mediated endocytosis pathway which normally internalises specific macromolecules. These vectors posess two distinct functional domains, a DNA-binding polycation such as polylysine, which is also necessary to condense the DNA, and a ligand domain for a specific cell surface receptor. This system has several potential advantages, including specific targeting determined by the ligand domain, and lack of constraint on DNA size. Unfortunately, efficiency of gene transfer *in vitro* has in general been low and appears to reflect lysosomal degradation of DNA following internalisation.

Adenoviral entry to cells is similar in many ways, except that acidification of the endosome results in disruption, allowing virions to proceed to the nucleus to complete their life cycle. Endosomal disruption is independent of viral gene expression, and is a direct function of viral capsid protein. Conjugation of adenovirus capsid via hexon protein to the DNA-polylysine complex utilises the endosomal disruption properties of the capsid, and enhances gene transfer by the molecular conjugate (Cotten et al. 1992). Ternary complexes in which selective ablation of adenovirus-mediated receptor binding to the cell is associated with retention of endosomal disruption properties of the capsid, and a distinct alternative ligand domain, may permit efficient gene transfer by molecular conjugates to specifically targeted cells.

Problems associated with immunogenicity and toxicity of the adenoviral capsid currently limit the application of this technology to cell culture. However, the principle of ligand mediated DNA delivery is attractive, and may in future become the gene transfer method of choice.

8.4 EXPRESSION OF TRANSFERRED GENES

Expression of transferred genes must be sufficient to correct both biochemical and clinical phenotype, and must be sustained for the functioning life of the cell. The necessity for tissue and differentiation-specific regulation will depend on the gene in

question, but for components of the NADPH-oxidase is largely unknown. Animal models of disease, generated by gene targeting, will help determine these parameters. Expression from heterologous promoters, for example the MLV LTR, may be sufficient, but may also be unpredictable, dependent on integration site, and may be susceptible to repression or 'shut-off' *in vivo.* Elucidation of natural regulatory mechanisms, and incorporation of relevant sequences in gene transfer vectors is therefore desirable.

8.4.1 Levels of expression required for reconstitution of the NADPH-oxidase

An estimate of the level of functional NADPH-oxidase reconstitution required for clinical cure can be ascertained from the study of 'variant' CGD patients. These patients invariably have X-linked disease, express normal or low levels of dysfunctional flavocytochrome, and may express up to 30% of normal NADPH-oxidase activity per cell. However, the pattern and severity of disease is often indistinguishable from the classical phenotype. Recently two kindreds with classical disease have been reported to have mutations within the promoter region of the $gp91^{phox}$ gene which resulted in undetectable expression of flavocytochrome in 90-95% of neutrophils, but normal expression in the remainder (Newberger et al. 1994). Surprisingly, this cell population was not defined by the pattern of X-inactivation, and it is difficult to reconcile the molecular lesions with both the cellular distribution of biochemical phenotype, and the observed clinical phenotype. In contrast to these two isolated kindreds, female carriers of classical X-linked disease with a predominance of abnormal neutrophils in the peripheral blood are usually completely unaffected. A tentative prediction from these observations would be that high level expression in a small but significant fraction of the total number of cells is preferable to partial activity in all cells. This has important implications for design of gene transfer vectors. Development of murine models of disease will be of particular use in determining levels of reconstitution necessary for phenotypic correction (Pollock et al. 1995).

The level of expression of individual NADPH-oxidase components required to reconstitute maximal enzymatic activity in mature neutrophils and monocytes is unknown. Some information can be obtained from cell-free studies in which cytosolic extracts are supplemented with recombinant protein (Chetty et al. 1995). In these

studies, the activity of B cell cytosol approaches that of neutrophil cytosol, and can be enhanced in a dose dependent manner by addition of recombinant $p67^{phox}$. Activity of neutrophil-derived cytosol can be enhanced in a similar way. This suggests that $p67^{phox}$ is the primary limiting component, and ties in well with the observation that expression of maximal cytosolic activity during the induction of HL60 cells is likewise dependent on the levels of $p67^{phox}$ (Levy et al. 1990). Experimental evidence from cell-free analysis of membranes derived from uninduced HL60 cells, and from genetic reconstitution of a flavocytochrome-negative PLB-985 XO myeloid cell line (Zhen et al. 1993), indicates that only a proportion of membrane-bound flavocytochrome is metabolically active at any one time, and that moderate levels of protein expression (<20%) are required to restore full activity. It therefore seems likely that for myeloid cells, $p67^{phox}$ is the primary limiting component, and that partial reconstitution of other components may be sufficient to support maximal NADPH-oxidase activity in deficient cells.

More recently, both bone marrow and cytokine-mobilised peripheral blood CD34+ cells derived from patients with CGD have been used as targets for retrovirus-mediated gene transfer (Sekhsaria et al. 1993; Li et al. 1994). Restoration of NADPH-oxidase function to progeny myeloid cells maintained in liquid culture occurred at low levels, and to myeloid colonies grown from clonogenic progenitors in semisolid methylcellulose at low frequency, which at least suggests that the Mo-MLV or the MPSV LTR may be adequate for expression in these cells. These experiments by their design measure transduction of relatively late progenitor cells.

8.5 PROSPECTS FOR GENE THERAPY OF CGD

The studies presented in this thesis demonstrate that biochemical function can be restored to cells derived from patients with autosomal recessive CGD, and go some way to define the mechanisms that are responsible for differentiation and tissue-specific expression of the $p47^{phox}$ gene. At present, efficient gene transfer to the optimal target cell, the pluripotent haematopoietic stem cell, is limited by the failure of the best developed gene transfer system based on murine leukaemia viruses to transduce

quiescent cells. Development of alternative systems based on adeno-associated virus may overcome this problem, but at present is hindered by the difficulty in producing high titre virus, free from contaminating adenovirus. Efficacy of novel vector systems for haematopoietic gene transfer must be assessed at the level of stem cell transduction. Unfortunately, current assay systems *in vitro* for gene transfer to progenitor cells do not adequately represent the PHSC population. Construction of animal models of haematopoiesis in immunodeficient mice are therefore necessary to test gene transfer to the most primitive cells, and may obviate the need for studies in non-human primates.

To be effective, successful and stable gene transfer must support levels of gene expression sufficient to cure the disease. Characterisation of natural promoter elements may ultimately allow fully regulated expression, but may not be necessary for non-toxic genes with simple regulatory patterns. In this situation viral promoters may be adequate, but may be particularly susceptible to position effect, and to shut-off *in vivo*. Expression of 'new'proteins to which the host immune system has never been tolerised may also result in diminished therapeutic benefit.

Gene therapy of CGD and other inherited haematopoietic disorders requires a much clearer understanding of stem cell biology, or the development of efficient gene transfer vehicles which are not dependent on cell-cycle. Problems of sustained expression will undoubtedly be encountered, and necessitates identification of natural regulatory mechanisms. Ultimately, delivery of large pieces of DNA on artificial chromosomes incorporating complete expression domains, or correction of genetic mutation by homologous recombination may obviate problems encountered with current vector systems.

8.6 CONCLUSIONS

- *Chronic granulomatous disease is a suitable candidate disorder for the application of somatic gene therapy.*

- *Utilisation of natural promoter elements may permit both differentiation and tissue specific regulation of transgenes. Preliminary analysis of the $p47^{phox}$ proximal promoter suggests that important elements lie outside the sequenced region.*

- *Defective NADPH-oxidase function can be reconstituted in immortalised CGD B cells by retrovirus-mediated gene transfer. The ability of similar vectors to transduce PHSCs may be limited by a requirement for cell division.*

- *Adeno-associated virus based vector systems transduce immortalised B cells at low frequency and may overcome some limitations of current murine retrovirus-based systems. At present, recombinant AAV preparations are of low titre, and are invariably contaminated by wtAd.*

- *rAAV can be recovered from E1-deleted adenoviral vectors, obviating the requirement for wtAd. A similar mechanism for recovery of the rAAV genome may be applicable to transduction of target cells.*

- *Adenoviral vectors encoding components of the NADPH-oxidase complement the genetic deficiency in primary CGD monocytes. This offers a rapid means for molecular diagnosis, and may be useful therapeutically.*

REFERENCES

Adam, M.A., and Miller, A.D. (1988) Identification of a signal in a murine retrovirus that is sufficient for packaging of non-retroviral RNA into virions. *J. Virol.* **62**, 3802-6.

Albritton, L.M., Tseng, L., Scadden, D., and Cunningham, J.M. (1989) A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. *Cell* **57**, 659-666.

Alexander, I.E., Russell, D.W., and Miller, A.D. (1994) DNA-damaging agents greatly increase the transduction of non-dividing cells by adeno-associated virus vectors. *J. Virol.* **68**, 8282-8287.

Allan, M., Lanyon, W.G., and Paul, J. (1983) Multiple origins of transcription in the 4.5kb upstream of the ε-globin gene. *Cell* **35**, 187-197.

Allan, M., and Paul, J. (1984) Transcription in vivo of an Alu family member upstream from the ε-globin gene. *Nucl. Acids. Res.* **12**, 1193-1200.

Alton, E.W.F.W., Middleton, P.G., Caplen, N.J., Smith, S.N., Steel, D.M., Munkonge, F.M., Jeffery, P.K., Geddes, D.M., Hart, S.L., Williamson, R., Fasold, K.I., Miller, A.D., Dickinson, P., Stevenson, B.J., McLachlan, G., Dorin, J.R., and Porteus, D.J. (1993) Non-invasive liposome-mediated gene delivery can correct the ion transport defect in cystic fibrosis mutant mice. *Nat. Genet.* **5**, 135-142.

Ament, M.E., and Ochs, H.D. (1973) Gastrointestinal manifestations of chronic granulomatous disease. *N. Engl. J. Med.* **288**, 382-387.

Amezaga, M.A., Bazzoni, F., Sorio, C., Rossi, F., and Cassatella, M.A. (1992) Evidence for the involvement of distinct signal transduction pathways in the regulation of constitutive and interferon γ-dependent gene expression of NADPH oxidase components (gp91-*phox*, p47-*phox*, and p22-*phox*) and high affinity receptor for IgG (FcγR-I) in human polymorphonuclear leukocytes. *J. Biol. Chem.* **79**, 735-744.

Anonymous (1964) Changes in the metabolic pattern of polymorphonuclear leukocytes during phagocytosis. *Br. J. Exp. Pathol* **45**, 548-559.

Antequera, F., Macleod, D., and Bird, A.P. (1989) Specific protection of methylated CpGs in mammalian nuclei. *Cell* **58**, 509-517.

Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C.G. and Segal, A.W. (1991) The small GTP-binding protein, p21rac 1, is involved in activation of the NADPH oxidase. *Nature* **353**, 668-670.

Abo, A., Boyhan, A., West, I., Thrasher, A.J. and Segal, A.W. (1992) Reconstitution of neutrophil NADPH oxidase activity in the cell-free system by four components: p67-*phox*, p47-*phox*, p21rac1, and cytochrome b_{-245}. *J. Biol. Chem.* **267**, 16767-16770

Abo, A. and Pick, E. (1991) Purification and characterization of a third cytosolic component of the superoxide-generating NADPH Oxidase of macrophages. *J. Biol. Chem.* **266**, 23577-23585.

Arella, M., Garzon, S., Bergeron, J., and Tijsson, P. (1990) Physicochemical properties, production, and purification of parvoviruses. In: *Handbook of parvoviruses, Volume 1*, (Tijssen, P. Ed) CRC Press, Florida 11-30.

Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973) Biological defence mechanisms: the production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* **52**, 741-744.

Babior, B.M., Kuver, R. and Curnutte, J.T. (1988) Kinetics of activation of the respiratory burst oxidase in a fully soluble system from human neutrophils. *J. Biol. Chem.* **263**, 1713-1718.

Bach, F.H., Albertini, R.J., Anderson, J.L., et al. (1968) Bone marrow transplantation in a patient with the Wiscott-Aldrich syndrome. *Lancet* **2**, 1364-1366.

Badwey, J.A., Curnutte, J.T., Robinson, J.M., Lazdins, J.K., Briggs, R.T., Karnovsky, M.J. and Karnovsky, M.L. (1980) Comparative aspects of oxidative metabolism of neutrophils from human blood and guinea pig peritonea: magnitude of the respiratory burst, dependence upon stimulating agents, and localization of the oxidases. *J Cell. Physiol.* **105**, 541-545.

Badwey, J.A. and Karnovsky, M.L. (1979) Production of superoxide and hydrogen peroxide by an NADH-oxidase in guinea pig polymorphonuclear leukocytes. Modulation by nucleotides and divalent cations. *J. Biol. Chem.* **254**, 11530-11537.

Baehner, R.L. and Karnovsky, M.L. (1968) Deficiency of reduced nicotinamide-adenine dinucleotide oxidase in chronic granulomatous disease. *Science* **162**, 1277-1279.

Baehner, R.L., Johnston R.B., Nathan, D.G. (1972) Comparative study of the metabolic and bactericidal characteristics of severely glucose-6-phosphate dehydrogenase deficient polymorphonuclear leukocytes and leukocytes from children with chronic granulomatous disease. *J. Reticuloendothel. Soc.* **12**, 150.

Baggiolini, M., Boulay, F., Badwey, J.A. and Curnutte, J.T. (1993) Activation of neutrophil leukocytes: chemoattractant receptors and respiratory burst. *FASEB* **7**, 1004-1010.

Baldridge, C.W. and Gerard, R.W. (1933) The extra respiration of phagocytosis. *Am. J Physiol.* **103**, 235-236.

Barklis, E., Mulligan, R.C., and Jaenisch, R. (1986) Chromosomal position or virus mutation permits retrovirus expression in embryonal carcinoma cells. *Cell* **47**, 391-399.

Barrett, J. (1992) Graft versus host disease. In: *Bone marrow transplantation in practice*, (Treleaven, J. and Barrett, J. Eds) Churchill Livingstone, 257-272.

Bar-Sagi, D., Rotin, D., Batzer, A., Mandiyan, V. and Schlessinger, J. (1993) SH3 domains direct cellular localisation of signalling molecules. *Cell* **74**, 83-91.

Battat, L. and Francke, U. (1989) Nsi I RFLP at the X-linked chronic granulomatous disease Locus (CYBB). *Nucleic Acids Res.* **17**, 3619.

Beato, M. (1989) Gene regulation by steroid hormones. *Cell* **56**, 335-344.

Beaton, A., Palumbo, P., and Berns, K.I. (1989) Expression from the adeno-associated virus p5 and p19 promoters is negatively regulated in trans by the Rep protein. *J. Virol.* **63**, 4450-4454.

Berardi, A.C., Wang, A., Levine, J.D., Lopez, P., and Scadden, D.T. (1995) Functional isolation and characterisation of human haematopoietic stem cells. *Science* **267**, 104-108.

Berendes, H., Bridges, R.A. and Good, R.A. (1959) A fatal granulomatous disease of childhood. The clinical, pathological, and laboratory features of a new syndrome. *Am. J. Dis. Child* **97**, 387.

Berenson, R.J., Andrews, R.G., Bensinger, W.I. Kalamasz, D., Knitter, G., Buckner, C.D., and Bernstein, I.D. (1988) Antigen CD34+ marrow cells engraft lethally irradiated baboons. *J. Clin. Invest.* **81**, 951-955.

Berenson, R.J., Bensinger, W.I., Hill, R.S., et al. (1991) Engraftment after infusion of CD34+ marrow cells in patients with breast cancer or neuroblastoma. *Blood* **77**, 1717-1722.

Berkner, K.L. (1992) Expression of heterologous sequences in adenoviral vectors. *Curr. Top. Microbiol. Immunol.* **158**, 39-66.

Bestor, T.H., and Coxon, A. (1993) The pros and cons of DNA methylation. *Curr. Biol.* **6**, 384-386.

Bett, A.J., Prevec, L., and Graham, F.L. (1993) Packaging capacity and stability of human adenovirus type 5 vectors. *J. Virol.* **67**, 5911-5921.

Bienzle, D., Abrams-Ogg, A.C.G., Kruth, S.A., Ackland-Snow, J., Carter, R.F., Dick, J.E., Jacobs, R.M., Kamel-Reid, S., and Dube, I. (1994) Gene transfer into hematopoietic stem cells: long term maintenance of in vitro activated progenitors without marrow ablation. *Proc. Natl. Acad. Sci. USA* **91**, 350-354.

Bird, A.P. (1986) CpG-rich islands and the function of DNA methylation. *Nature* **321**, 209-213.

Blacklow, N.R., Hoggan, M.D., and Rowe, W.P. (1968) Serologic evidence for human infection with adeno-associated viruses. *J. Natl. Cancer Inst.* **40**, 319.

Blackwell, T.K., and Weintraub, H. (1990) DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. *Science* **250**, 1104-10.

Bodine, D.M., Moritz, T., Donahue, R.E., Luskey, B.D., Kessler, S.W., Martin, D.I.K., Orkin, S.H., Neinhuis, A.W., and Williams, D.A. (1993) Long term in vivo expression of a murine adenosine deaminase gene in rhesus monkey hematopoietic cells of multiple lineages after retroviral-mediated gene transfer into CD34+ bone marrow cells. *Blood* **82**, 1975-1980.

Bohler, M.C., Seger, R.A., Mouy, R., Vilmer, E., Fischer, A. and Griscelli, C. (1986) A study of 25 patients with chronic granulomatous disease: a new classification by correlating respiratory burst, cytochrome b, and flavoprotein. *J Clin. Immunol.* **6**, 136-145.

Bokoch, G.M., Quilliam, L.A., Bohl, B.P., Jesaitis, A.J. and Quinn, M.T. (1991) Inhibition of Rap1A-binding to cytochrome b_{558} of NADPH Oxidase by phosphorylation of Rap1A. *Science* **254**, 1794-1796.

Bolscher, B.G.J.M., van Zwieten, R., Kramer, I.M., Weening, R.S., Verhoeven, A.J. and Roos, D. (1989) A phosphoprotein of Mr 47,000 defective in autosomal CGD, co-purifies with one of 2 soluble components required for NADPH:O_2 oxido-reductase activity in human neutrophils. *J. Clin. Invest* **83**, 757-763.

Bolscher, B.G.J.M., Denis, S.W., Verhoeven, A.J. and Roos, D. (1990) The activity of one soluble component of the cell-free NADPH:O_2 Oxido-reductase of human neutrophils depends on guanosine 5'-O-(3-Thio)triphosphate. *J. Biol. Chem.* **265**, 15782-15787.

Bolscher, B.G.J.M., de Boer, M., De Klein, A., Weening, R.S. and Roos, D. (1991) Point mutations in the β-Subunit of cytochrome b_{558} leading to X-linked chronic granulomatous disease. *Blood* **77**, 2482-2487.

Bonifer, C., Yannoutsos, N., Kruger, G., Grosveld, F., and Sippel. A.E. (1994) Dissection of the locus control function located on the chicken lysozyme gene domain in trasgenic mice. *Nucl. Acids. Res.* **22**, 4202-4210.

Bottinger, E.P., Shelley, C.S., Farokhzad, O.C. and Arnaout, M.A. (1994) The human beta 2 integrin CD18 promoter consists of two inverted Ets cis elements. *Mol. Cell. Biol.* **14**, 2604-2615.

Boyes, J., and Bird, A.P. (1991) DNA methylation inhibits transcription indirectly via a methyl CpG-binding protein. *Cell* **64**, 1123-1134.

Breathnach, R., and Chambon, P. (1981) Organisation and expression of eukaryotic split genes coding for proteins. *Ann. Rev. Biochem.* **50**, 349-83.

Brenner, M.K., Rill, D.R., Holladay, M.S., Heslop, H.E., Moen, R.C., Buschle, M., Krance, R.A., Santana, V.M., Anderson, W.F., and Ihle, J.N. (1993a) Gene marking to determine whether autologous marrow infusion restores long-term haematopoiesis in cancer patients. *Lancet* **342**, 1134-1137.

Brenner, M.K., Rill, D.R., H.E., Moen, R.C., Krance, R.A., Mirro, J., Anderson, W.F., and Ihle, J.N. (1993b) Gene marking to trace origin of relapse after autologous bone-marrow transplantation. *Lancet* **341**, 85-86.

Bromberg, Y. and Pick, E. (1984) Unsaturated fatty acids stimulate NADPH-dependent superoxide production by a cell-free system derived from macrophages. *Cell Immunol.* **88**, 213-221.

Bromberg, Y. and Pick, E. (1985) Activation of NADPH-dependent superoxide production in a cell-free system by sodium dodecyl sulfate. *J. Biol. Chem.* **260**, 13539-13545.

Brown, K.E., Anderson, S.M., and Young, N.S. (1993) Erythrocyte P antigen: cellular receptor for B19 parvovirus. *Science* **262**, 114-116.

Buescher, E.S., and Gallin, J.I. (1992) Leukocyte transfusions in chronic granulomatous disease: persistence of transfused leukocytes in sputum. *N.Engl.J.Med* **307**, 800-803.

Bukrinsky, M.I., Haggerty, S., Dempsey, M.P., Sharova, N., Adzhubei, A., Spitz, L., Lewis, P., Goldfarb, D., Emerman, M., and Stenevenson,M. (1993) A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature* **365**, 666-669.

Burbach, J.P., Lopes da Silva, S., Cox, J.J., Adan, R.A., Cooney, A.J., Tsai, M.J. and Tsai, S.Y. (1994) Repression of estrogen-dependent stimulation of the oxytocin gene by chicken ovalbumin upstream promoter transcription factor I. *J. Biol. Chem.* **269**, 15046-15053.

Busslinger, M., Hurst, J., and Flavell, R.A. (1983) DNA methylation and the regulation of globin gene expression. *Cell* **34**, 197-206.

Cagan, R.H. and Karnovsky, M.L. (1964) Enzymic basis of the respiratory stimulation during phagocytosis. *Nature* **204**, 255-257.

Caldwell, S.E., McCall, C.E., Hendricks, C.L., Leone, P.A., Bass, D.A. and Phail, L.C. (1988) Co-regulation of NADPH oxidase activation and phosphorylation of a 48-kD protein(s) by a cytosolic factor defective in autosomal recessive chronic granulomatous disease. *J Clin. Invest.* **81**, 1485-1496.

Calabretta, B., Robberson, D.L., Maizel, A.L., and Saunders, G.F. (1981) mRNA in human cells contains sequences complementary to the Alu family of repeated DNA. *Proc. Natl. Acad. Sci. USA* **78**, 6003-6007.

Carter, B.J., Laughlin, C.A., De La Maza, L.M., and Myers, M. (1979) Adeno-associated virus autointerference. *Virology* **92**, 449-462.

Carter, B.J. (1990a) The growth cycle of adeno-associated virus. In: *Handbook of parvoviruses, Volume 1*, (Tijssen, P. Ed) CRC Press, Florida 155-168.

Carter, B.J., Mendelson, E., and Trempe, J.P. (1990b) AAV DNA replication, integration, and genetics. In: *Handbook of parvoviruses, Volume 1*, (Tijssen, P. Ed) CRC Press, Florida 169-226.

Casimir, C.M., Bu-Ghanim, H.N., Rodaway, A.R.F., Bentley, D.L., Rowe, P. and Segal, A.W. (1991) Autosomal recessive chronic granulomatous disease caused by deletion at a dinucleotide repeat. *Proc. Natl. Acad. Sci. USA* **88**, 2753-2757.

Casimir, C.M., Chetty, M., Bohler, M., Garcia, R., Fischer, A., Griscelli, C., Johnson, B. and Segal, A.W. (1992) Identification of the defective NADPH-oxidase component in chronic granulomatous disease: a study of 57 European families. *Eur. J Clin. Invest.* **22**, 403-406.

Cassatella, M.A., Hartman, L., Perussia, B., and Trinchieri, G. (1989) Tumour necrosis factor and immune interferon synergistically induce cytochrome b heavy chain gene expression and NADPH-oxidase in human leukemic myeloid cells. *J. Clin. Invest.* **83**, 1570-1579.

Cassatella, M.A., Bazzoni, F., Flynn, R.M., Dusi, S., Trinchieri, G., and Rossi, F. (1990) Molecular basis of interferon-γ and lipopolysaccharide enhancement of phagocyte respiratory burst capability. *J. Biol. Chem.* **265**, 20241-20246.

Cassatella, M.A., Bazzoni, F., Calzetti, F., Guasparri, I, Rossi, F., Trinchieri, G. (1991) Interferon-γ transcriptionally modulates the expression of the genes for the high affinity IgG-Fc receptor and the 47kDa cytosolic component of the NADPH-oxidase in human polymorphonuclear leukocytes. *J. Biol. Chem.* **266**, 22079-22082.

Casto, B.C., Armstrong, J.A., Atchison, R.W., and Hammon, W.McD. (1967) Studies on the relationship between adeno-associated virus type 1 (AAV-1) and adenoviruses. I and II. *Virology* **32**, 52-59, and **33**, 452-458.

Cech, P. and Lehrer, R.I. (1984) Phagolysosomal pH of human neutrophils. *Blood* **63**, 88-95.

Cedar, H. (1988) DNA methylation and gene activity. *Cell* **53**, 3-4.

Cepko, C., Roberts, B., and Mulligan, R. (1984) Construction and applications of a highly transmissible murine retrovirus shuttle vector. *Cell* **37**, 1053-1062.

Chanock, S.J., Barrett, D.M., Curnutte, J.T. and Orkin, S.H. (1991) Gene structure of the cytosolic component, p47-*phox* and mutations in autosomal recessive chronic granulomatous disease. *Blood* **78**, 165a.

Chanock, S.J., Faust, L.R., Barrett, D., Bizal, C., Maly, F.E., Newburger, P.E., Ruedi, J.M., Smith, R.M., and Babior, B.M. (1992) Superoxide production by B lymphocytes lacking the respiratory burst oxidase subunit p47*phox* after transfection with an expression vector encoding a p47*phox* cDNA. *Proc. Natl. Acad. Sci. USA* **89**, 10174-10177.

Cavalier-Smith, T. (1974) Palindromic base sequences and replication of eukaryotic chromosome ends. *Nature* **250**, 467-470.

Chejanovsky, N., and Carter, B.J. (1989) Mutagenesis of an AUG codon in the adeno-associated virus rep gene: effects on viral DNA replication. *Virology* **173**, 120-128.

Chen, C., and Okayama, H. (1987) High efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**, 2745-2752.

Chetty, M., Thrasher, A.J., Abo, A., Casimir, C.M. (1995) Low NADPH oxidase activity in EBV-immortalised B lymphocytes is due to a post-transcriptional block in expression of cytochrome b_{558}. *Biochem. J.* **306**. 141-145.

Chiba, T., Kaneda, M., Fujii, H., Clark, R.A. and Nauseef, W.M. (1990) Two cytosolic components of the neutrophil NADPH oxidase, p47-*phox* and p67-*phox*, are not flavoproteins. *Biochem. Biophys. Res. Comm.* **173**, 376-381.

Chin, T.W., Stiehm, E.R., Falloon, J. and Gallin, J.I. (1987) Corticosteroids in treatment of obstructive lesions of chronic granulomatous disease. *J. Pediatr* **111**, 349-352.

Chiorini, J.A., Wiener, S.M., Owens, R.A., Kyostio, S.R.M., Kotin, R.M., and Safer, B. (1994) Sequence requirements for stable binding and function of Rep68 on the adeno-associated virus type 2 inverted terminal repeats. *J. Virol.* **68**, 7448-7457.

Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Chung, J.H., Whiteley, M., and Felsenfeld, G. (1993) A 5' element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in Drosophila. *Cell* **74**, 505-514.

Clark, R.A., Malech, H.L., Gallin, J.I., Nunoi, H., Volpp, B.D., Pearson, D.W., Nauseef, W.M. and Curnutte, J.T. (1989) Genetic variants of chronic granulomatous disease: prevalence of deficiencies of two cytosolic components of the NADPH oxidase system. *N. Engl. J Med.* **321**, 647-652.

Clark, R.A., Volpp, B.D., Leidal, K.G. and Nauseef, W.M. (1990) Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. *J. Clin. Invest.* **85**, 714-721.

Cloyd, M.W., Hartley, J.W., and Rowe, W.P. (1982) Lymphomagenicity of recombinant mink cell focus-inducing murine leukemia viruses. *J. Exp. Med.* **151**, 542-552.

Coffin, J.M. (1992) Structure and classification of retroviruses. In: *The Retroviridae, Volume 1,* (Levy, J.A. Ed.) Plenum Press, New York, 19-49.

Cohen-Tanugi, L., Morel, F., Pilloud-Dagher, M., Seigneurin, J.M., Francois, P., Bost, M., and Vignais, P.V. (1991) Activation of O_2-generating oxidase in an heterologous cell-free system derived from Epstein-Barr-virus-transformed human B lymphocytes and bovine neutrophils. *Eur. J. Biochem.* **202**, 649-655.

Cone, R., and Mulligan, R. (1984) High efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host-range. *Proc. Natl. Acad. Sci. USA* **81**, 6349-6353.

Cooney, A.J., Tsai, S.Y., O'Malley, B.W. and Tsai, M.J. (1991) Chicken ovalbumin upstream promoter transcription factor binds to a negative regulatory region in the human immunodeficiency virus type 1 long terminal repeat. *J. Virol.* **65**, 2853-2860.

Cooney, A.J., Tsai, S.Y., O'Malley, B.W. and Tsai, M.J. (1992) Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCA response elements, allowing COUP-TF to repress hormonal induction of the vitamin D3, thyroid hormone, and retinoic acid receptors. *Mol. Cell Biol.* **12**, 4153-4163.

Cooney, A.J., Leng, X., Tsai, S.Y., O'Malley, B.W. and Tsai, M.J. (1993) Multiple mechanisms of chicken ovalbumin upstream promoter transcription factor-dependent repression of transactivation by the vitamin D, thyroid hormone, and retinoic acid receptors. *J. Biol. Chem.* **268**, 4152-4160.

Cooper, D.N., and Youssoufian, M. (1988) The CpG dinucleotide and human genetic disease. *Hum. Genet.* **78**, 151-155.

Cooper, M.R., DeChatelet, L.R., McCall, L.R., LaVia, M.F., Spurr, C.L., and Baehner, R.L. (1972) Complete deficiency of leukocyte glucose-6-phosphate dehydrogenase with defective bactericidal activity. *J. Clin. Invest.* **51**, 769.

Correll, P.H., and Karlsson, S. (1994) Towards therapy of Gauchers disease by gene transfer into haematopoietic cells. *Eur. J. Haematol.* **53**, 253-264.

Cotten, M., Wagner, E., Zatloukal, K., Phillips, S., Curiel, D.T., and Birnstiel, M.L. (1992) High efficiency receptor-mediated delivery of small and large (48 kilobase) gene constructs using the endosome-disruption activity of defective or chemically inactivated adenoviral particles. *Proc. Natl. Acad. Sci. USA* **89**, 6094-6098.

Cournoyer, D., and Caskey, C.T. (1993) Gene therapy of the immune system. *Annu. Rev. Immunol.* **11**, 297-329.

Craigie, R., Fujiwara, T., and Bushman, F. (1990) The IN protein of Moloney Murine Leukemia Virus processes the viral DNA ends and accomplishes their integration in vitro. *Cell* **62**, 829-837.

Cross, A.R., Higson, F.K., Jones, O.T., Harper, A.M. and Segal, A.W. (1982a) The enzymic reduction and kinetics of oxidation of cytochrome b-245 of neutrophils. *Biochem. J* **204**, 479-485.

Cross, A.R., Jones, O.T., Garcia, R. and Segal, A.W. (1982b) The association of FAD with the cytochrome b_{-245} of human neutrophils. *Biochem. J.* **208**, 759-763.

Cross, A.R. and Jones, O.T.G. (1991) Enzymic mechanisms of superoxide production. *Biochim. Biophys. Acta* **1057**, 281-298.

Curnutte, J.T. (1985) Activation of human neutrophil nicotinamide adenine dinucleotide phosphate reduced (triphosphopyridine nucleotide, reduced) oxidase by arachidonic acid in a cell-free system. *J Clin. Invest.* **75**, 1740-1743.

Curnutte, J.T., Kuver, R. and Babior, B.M. (1987) Activation of the respiratory burst oxidase in a fully soluble system from human neutrophils. *J. Biol. Chem.* **262**, 6450-6452.

Curnutte, J.T., Berkow, R.L., Roberts, R.L., Shurin, S.B. and Scott, P.J. (1988) Chronic granulomatous disease due to a defect in the cytosolic factor required for nicotinamide adenine dinucleotide phosphate oxidase activation. *J Clin. Invest.* **81**, 606-610.

Curnutte, J.T. (1993) Chronic granulomatous disease: The solving of a clinical riddle at the molecular level. *Clin. Immunol. Immunopathol.* **67**, S2-S15.

Curnutte, J.T., Erickson,R.W., Ding, J., and Badwey, J.A. (1994) Reciprocal interactions between protein kinase C and components of the NADPH-oxidase complex may regulate superoxide production by neutrophils stimulated with a phorbol ester. *J. Biol. Chem.* **269**, 10813-10819.

Cuypers, H.T., Selten, G., Quint, W., Zijlstra, M., Maandag, E.R., Boelens, W., Van Wezenbeek, P., Melief, C., and Berns, A. (1984) Murine leukemia virus-induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal region. *Cell* **37**, 141-150.

Dalton, W.T., Ahearn, M.J., McCredie, K.B., Freireich, E.J., Stass, S.A., and Trujillo, J.M. (1988) HL-60 cell line was derived from a patient with FAB-M2 not FAB-M3. *Blood* **71**, 242-247.

Dancis, A., Roman, D.G., Anderson, G.J., Hinnebusch, A.G. and Klausner, R.D. (1992) Ferric reductase of saccharomyces cerevisiae: molecular characterisation, role in iron uptake and transcriptional control by iron. *Proc. Natl. Acad. Sci. USA* **3869**, 3873

Danos, O., and Mulligan, R. (1988) Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc. Natl. Acad. Sci. USA* **85**, 6460-6464.

de Boer, M., Bolscher, B.G.J.M., Dinauer, M.C., Orkin, S.H., Smith, C.I.E., Ahlin, A., Weening, R.S. and Roos, D. (1992a) Splice site mutations are a common cause of X-linked chronic granulomatous disease. *Blood* **80**, 1553-1558.

de Boer, M., De Klein, A., Hossle, J., Seger, R., Corbeel, L., Weening, R.S. and Roos, D. (1992b) Cytochrome b_{558}-negative, autosomal recessive chronic granulomatous disease; two new mutations in the cytochrome b_{558} light chain of the NADPH oxidase (p22-*phox*). *Am. J. Hum. Genet.* **51,**1127-1135.

de Boer, M., Hilarius, P.M., Hossle, J.P., Verhoeven, A.J., Graf, N., Kenney, R.T., Seger, R., and Roos, D. (1994) Autosomal recessive chronic granulomatous disease with absence of the 67kD NADPH oxidase component: identification of mutation and detection of carriers. *Blood* **2**, 531-536.

Del Maestro, R., Thaw, H.H., Bjork, J., Planker, M. and Arfors, K.E. (1980) Free radicals as mediators of tissue injury. *Acta Physiol. Scand. (Suppl.)* **492**, 43-57.

Dexter, T.M., Allen, T.D., and Lajtha, L.G. (1977) Conditions controlling the proliferation of hematopoietic stem cells *in vitro. J. Cell. Physiol.* **91**, 335.

Dick, J.E., Lapidot, T., and Pflumio, F. Transplantation of normal and leukemic human bone marrow into immune-deficient mice: development of animal models for human haematopoiesis (1991) *Immunol. Rev.* **124**, 25-43.

Dick, J.E. (1995) Evaluation of procedures for gene transfer into primitive hematopoietic cells using the SCID-repopulation assay. *J. Cell. Biochem.* supp **21A**, 354.

Didsbury, J., Weber, R.F., Bokoch, G.W., Evans, T. and Snyderman, R.J. (1989) Rac, a novel ras-related family of proteins that are botulinum toxin substrates. *J. Biol. Chem.* **264**, 16378-16382.

Dillon, N., and Grosveld, F. Transcriptional regulation of multigene loci: multilevel control (1993) *Trends Genet.* **9**, 134-137.

Dinauer, M.C., Orkin, S.H., Brown, R., Jesaitis, A.J. and Parkos, C.A. (1987) The glycoprotein encoded by the X-linked chronic granulomatous disease locus is a component of the neutrophil cytochrome b complex. *Nature* **327**, 717-720.

Dinauer, M.C., Pierce, E.A., Bruns, G.A.P., Curnutte, J.T. and Orkin, S.H. (1990) Human neutrophil cytochrome b light chain (p22-*phox*): gene structure, chromosomal location and mutations in cytochrome-negative autosomal recessive chronic granulomatous disease. *J. Clin. Invest.* **86**, 1729-1737.

Dinauer, M.C., Pierce, E.A., Erickson, R.W., Muhlebach, T.J., Messner, H., Orkin, S.H., Seger, R.A. and Curnutte, J.T. (1991) Point mutation in the cytoplasmic domain of the neutrophil p22-*phox* cytochrome b subunit is associated with a nonfunctional NADPH oxidase and chronic granulomatous disease. *Proc. Natl. Acad. Sci. USA* **88**, 11231-11235.

Donahue, R.E., Kessler, S.W., Bodine, D., McDunagh, K., Dunbar, C., Goodman, S., Agricola, B., Byrne, E., Raffeld, M., Moen, R. et al. (1992) Helper virus-induced T cell lymphoma in non-human primates after retroviral mediated gene transfer. *J. Exp. Med.* **176**, 1125-35.

Dorseuil, O., Vazquez, A., Lang, P., Bertoglio, J., Gacon, G., and Leca, G. (1992) Inhibition of superoxide production in B lymphocytes by Rac antisense oligonucleotides. *J.Biol.Chem.* **267,** 20540-20542.

Duncan, C.H., Jagadeeswaran, P., Wang, R.R.C., and Weissman, S.M. (1981) Structural analysis of templates and RNA polymerase III transcripts of Alu family sequences interspersed among the human β-like globin genes. *Gene* **13**, 185-196.

Dusi, S., Bianca, V.D., Grzeskowiak, M., and Rossi, F. (1993) Relationship between phosphorylation and translocation to the plasma membrane of p47*phox* and p67*phox* and activation of the NADPH oxidase in normal and Ca2+-depleted human neutrophils. *Biochem.J.* **290,** 173-178.

Eichbaum, Q.G., Iyer, R., Raveh, D.P., Mathieu, C. and Ezekowitz, R.A. (1994) Restriction of interferon gamma responsiveness and basal expression of the myeloid human Fc gamma R1b gene is mediated by a functional PU.1 site and a transcription initiator consensus. *J. Exp. Med.* **179**, 1985-1996.

Eklund, E.A., Marshall, M., Gibbs, J.B., Crean, C.D. and Gabig, T.G. (1991) Resolution of a low molecular weight G protein in neutrophil cytosol required for NADPH oxidase activation and reconstitution by recombinant Krev-1 Protein. *J. Biol. Chem.* **266**, 13964-13970.

Emerman, M., and Temin, H.M. (1984) Genes with promoters in retrovirus vectors can be independently suppressed by an epigenetic mechanism. *Cell* **39**, 449-467.

Engelhardt, J.F., Litzky, L., and Wilson, J.M. (1994) Prolonged transgene expression in cotton rat lung with recombinant adenoviruses defective in E2A. *Hum. Gene Ther.* **5**, 1217-1229.

Ezekowitz, R.A.B., Orkin, S.H. and Newberger, P.E. (1987) Recombinant interferon-gamma augments phagocyte superoxide production and X-chronic granulomatous

disease gene expression in X-linked variant chronic granulomatous disease. *J. Clin. Invest* **80**, 1009-1016.

Ezekowitz, R.A.B., Dinauer, M.C., Jaffe, H.S., Orkin, S.H. and Newburger, P.E. (1988) Partial correction of the phagocyte defect in patients with X-linked chronic granulomatous disease by subcutaneous interferon gamma. *N. Engl. J Med.* **319**, 146-151.

Ezekowitz, R.A.B. (1992) Chronic granulomatous disease: an update and a paradigm for the use of interferon-gamma as adjunct immunotherapy in infectious disease. *Curr. Top. Microbiol. Immunol.* **181**, 283-292.

Feinman, R., Qiu, W.Q., Pearse, R.N., Nikolajczyk, B.S., Sen, R., Sheffrey, M. and Ravetch, J.V. (1994) PU.1 and an HLH family member contribute to the myeloid-specific transcription of the FcγRIIIA promoter. *EMBO J.* **13**, 3852-3860.

Finn, A., Hadzic, N., Morgan, G., Strobel, S. and Levinsky, R.J. (1990) Prognosis of chronic granulomatous disease. *Arch. Dis. Child.* **65**, 942-945.

Fischer, A., Landais, P., Friedrich, W., Morgan, G., Gerritson, B., Fasth, A., Griscelli, C., Goldman, S.F., Levinsky, R.J., and Vossen, J. (1990) European experience of bone marrow transplantation for severe combined immunodeficiency. *Lancet* **ii**, 850-854.

Fischer, A., Segal, A.W., Seger, R. and Weening, R.S. (1993) The management of chronic granulomatous disease. *Eur. J. Pediatr* **152**, 896-899.

Flanagan, J.R., Becker, K.G., Ennist, D.L., Gleason, S.L., Driggers, P.H., Levi, B-Z., Appella, E., and Ozato, K. (1992) Cloning of a negative transcription factor that binds to the upstream conserved region of Moloney murine leukemia virus. *Mol. Cell. Biol.* **12**, 38-44.

Flotte, T.R., Afione, S.A., Solow, R., Drumm, M., Markakis, D., Guggino, W.B., Zeitlin, P.L., and Carter, B.J. (1993) Expression of the cystic fibrosis transmembrane conductance regulator from a novel adeno-associated virus promoter. *J. Biol. Chem.* **268**, 3781-3790.

Forrest, C.B., Forehand, J.R., Axtell, R.A., Roberts, R.L. and Johnston, R.B.J. (1988) Clinical features and current management of chronic granulomatous disease. In: *Hematology/Oncology Clinics of North America: Phagocytic defects II*, (Curnutte, J.T. Ed.) 253-266.

Fry, M.J. (1992) Defining a new GAP family. *Curr. Biol* **2**, 78-80.

Fujita, I., Takeshige, K. and Minakami, S. (1987) Characterization of the NADPH-dependent superoxide production activated by sodium dodecyl sulfate in a cell-free system of pig neutrophils. *Biochim. Biophys. Acta* **931**, 41-48.

Gabig, T.G. and Lefker, B.A. (1984) Deficient flavoprotein component of the NADPH-dependent O_2^- generating oxidase in the neutrophils from three male patients with chronic granulomatous disease. *J Clin. Invest.* **73**, 701-705.

Gallin, J.I.e. (1991) A controlled trial of interferon gamma to prevent infection in chronic granulomatous disease. *N. Engl. J. Med.* **324**, 509-516.

Garcia, R.C., Cross, A.R. and Segal, A.W. (1986) The development of cytochrome b_{-245} in maturing human macrophages. *Biochem. J.* **239**, 647-651.

Garcia, R.C. and Segal, A.W. (1984) Changes in the subcellular distribution of the cytochrome b_{-245} on stimulation of human neutrophils. *Biochem. J.* **219**, 233-242.

Gatti, R.A., Meuwissen, J.J., Allen, H.D., Hong, R., and Good, R.A. (1968) Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet* **i**, 1366-1369.

Gautsch, J., and Wilson, M. (1983) Delayed de novo methylation in teratocarcinoma suggests additional tissue-specific mechanisms for controlling gene expression. *Nature* **301**, 32-37.

Giraud, C., Winocour, E., and Berns, K.I. (1994) Site-specific integration by adeno-associated virus is directed by a cellular DNA sequence. *Proc. Natl. Acad. Sci. USA* **91**, 10039-10043.

Golemis, E., Li, Y., Frederickson, T.N., Hartley, J.W., and Hopkins, N. (1989) Distinct segments within the enhancer region collaborate to specify the type of leukemia induced by non-defective Friend and Moloney virus. *J. Virol.* **63**, 328-337.

Gorlin, J. (1991) Identification of (CA/GT)n Polymorphisms within the X-linked chronic granulomatous disease (X-CGD) gene; utility for prenatal diagnosis. *Blood* **78**, 433a

Gout, I., Dhand, R., Hiles, I.D., Fry, M.J., Panayotou, G., Truong, O., Totty, N.F., Hsuan, J., Booker, G.W., Campbell, I.D. and Waterfield, M.D. (1993) The GTPase Dynamin binds to and is activated by a subset of SH3 domains. *Cell* **75**, 25-36.

Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. (1977) Characterisation of a human cell line transformed by DNA from human adenovirus 5. *J. Gen. Virol.* **36**, 59-72.

Gray, G.R., Stomatoyannopoulos, G., Naiman, S.C., Kilman, N.R., Klebanoff, S.J., Austin, T., Yoshida, A., and Robinson, G.C.F. (1973) Neutrophil dysfunction, chronic granulomatous disease and non-spherocytic hemolytic anemia caused by complete deficiency of glucose-6-phosphate dehydrogenase. *Lancet* **2**, 530.

Green, S. (1993) Promiscuous liasons. *Nature* **361**, 590-591.

Gross, D.S., and Garrard, W.T. (1988) Nuclease hypersensitive sites in chromatin. *Ann. Rev. Biochem.* **57**, 159-197.

Grosveld, F., Blom van Assendelft, G., Greaves, D., and Kollias, G. (1987) Position-independent high level expression of the human β globin gene in transgenic mice. *Cell* **51**, 975-985.

Grove, M., and Plumb, M. (1993) C/EBP, NF-kappa, and c-Ets family members and transcriptional regulation of the cell-specific and inducible macrophage inflammatory protein 1 alpha immediate-early gene. *Mol. Cell. Biol.* **13**, 5276-5289.

Haj-Ahmad, Y., and Graham, F.L. (1986) Development of a helper-independent human adenovirus vector and its use in the transfer of the herpes simplex virus thymidine kinase gene. *J. Virol.* **57**, 267-274.

Hall, A. (1992) Ras-related GTPases and the cytoskeleton. *Mol. Biol. Cell* **3**, 475-479.

Hajihosseini, M., Lavachev, L., and Price, J. (1993) Evidence that retroviruses integrate into post-replication host DNA. *EMBO J.* **12**, 4969-4974.

Hanecak, R., Pattengale, P.K., and Fan, H. (1991) Deletion of a GC-rich region flanking the enhancer element within the long terminal repeat sequences alters the disease specificity of Moloney murine leukemia virus. *J. Virol.* **65**, 5357-5363.

Hare, J.T., and Taylor, H. (1985) One role for DNA methylation in vertebrate cells in strand discrimination in mismatch repair. *Proc. Natl. Acad. Sci. USA* **82**, 7350-7354.

Harper, A.M., Dunne, M.J. and Segal, A.W. (1984) Purification of cytochrome b_{-245} from human neutrophils. *Biochem. J* **219**, 519-527.

Harper, A.M., Chaplin, M.F. and Segal, A.W. (1985) Cytochrome b_{-245} from human neutrophils is a glycoprotein. *Biochem. J* **227**, 783-788.

Harris, P., and Ralph, P. (1985) Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. *J. Leuk. Biol.* **37**, 407.

Hasui, M., Hattori, K., Taniuchi, S., Kohdera, U., Nishikawa, A., Kinoshita, Y. and Kobayashi, Y. (1993) Decreased CD4+CD29+ (memory-T) cells in patients with chronic granulomatous disease. *J. Infect. Dis.* **167**, 983-985.

Hattori, H. (1961) Studies on the labile, stable NADI oxidase and peroxidase staining reactions in the isolated particles of horse granulocytes. *Nagoya J Med Sci* **23**, 362-378.

Hatzoglou, M., Hodgson, C.P., Mularo, F., and Hanson, R.W. (1990) Efficient packaging of a specific VL30 retroelement by ψ2 cells which produce MoMLV recombinant retroviruses. *Hum. Gene Ther.* **1**, 385-397.

Hayakawa, H., Kobayashi, N. and Yata, J. (1985) Chronic granulomatous disease in Japan: a summary of the clinical features of 84 registered patients. *Acta Pediatr. Jpn* **27**, 1-140.

Heijnen, C.J., Van Der Meer, J.W.M. and Zegers, B.J.M. (1986) Altered antigen-presentation in the induction of the in-vitro antigen-specific T helper cell function in patients with chronic granulomatous disease. *Clin. Exp. Immunol* **66**, 111-117.

Heilbronn, R., Burkle, A., Stephen, S., and zur Hausen, H. (1990) the adeno-associated virus rep gene suppresses herpes simplex virus-induced DNA amplification. *J. Virol.* **64**, 3012-3018.

Hermonat, P.L., Labow, M.A., Wright, R., Berns, K.I., and Muzyczka, N. (1984) Genetics of adeno-associated virus: isolation and preliminary characterisation of adeno-associated virus type 2 mutants. *J. Virol.* **51**, 329-339.

Hermonat, P.L., and Muzyczka, N. (1984) Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammalian tissue culture cells. *Proc. Natl. Acad. Sci. USA* **81**, 6466-6470.

Hermonat, P.L. (1989) The adeno-associated virus Rep78 gene inhibits cellular transformation by bovine papillomavirus. *Virology* **172**, 253-261.

Heyman, R.A., Mangelsdorf, D.J., Dyck, J.A., Stein, R.B., Eichele, G., Evans, R.M., and Thaller, C. (1992) 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* **68**, 397-406.

Heyneman, R.A. and Vercauteren, R.E. (1984) Activation of a NADPH oxidase from horse polymorphonuclear leukocytes in a cell-free system. *J Leukocyte. Biol.* **36**, 751-759.

Heyworth, P.G., Shrimpton, C.F. and Segal, A.W. (1989) Localisation of the 47kDa phosphoprotein involved in the respiratory burst NADPH oxidase of phagocytic cells. Evidence for its translocation from the cytosol to plasma membrane. *Biochem. J* **260**, 243-248.

Heyworth, P.G., Curnutte, J.T., Nauseef, W.M., Volpp, B.D., Pearson, D.W., Rosen, H. and Clark, R.A. (1991) Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-*phox* and p67-*phox* requires interaction between p47-*phox* and cytochrome b_{558}. *J. Clin. Invest.* **87**, 352-356.

Heyworth, P.G. and Segal, A.W. (1986) Further evidence for the involvement of a phosphoprotein in the respiratory burst oxidase of human neutrophils. *Biochem. J* **239**, 723-731.

Hirt, B. (1967) Selective extraction of polyoma DNA from infected mouse cultures. *J. Mol. Biol.* **126**, 275-288.

Hoeben, R., Migchielson, A., van der Jagt, R., van Ormondt, H., and van der Eb, A. (1991) Inactivation of the Moloney murine leukemia virus long terminal repeat in murine fibroblast cell lines is associated with methylation, and dependent on its chromosomal position. *J. Virol.* **65**, 904-912.

Holscher, C., Horer, M., Kleinschmidt, J.A., Zentgraf, H., Burkle, A., and Heilbronn, R. (1994) Cell lines inducibly expressing the adeno-associated virus rep gene: requirments for productive replication of rep-negative AAV mutants. *J. Virol.* **68**, 7169-7177.

Holyoake, T.L., and Alcorn, M.J. (1994) CD34+ haematopoietic cells: biology and clinical applications. *Blood Reviews* **8**, 113-124.

Horwitz, M.S. (1990) Adenoviridae and their replication. In: *Virology* (Fields, B.N. Ed.), Raven Press Ltd., New York, 1679-1721.

Holliday, R., and Ho, T. (1990) Evidence for allelic exclusion in CHO cells. *New Biologist* **2**,719-726.

Hollon, T., and Yoshimura, F.K. (1989) Mapping of functional regions of murine retrovirus long terminal repeat enhancers: enhancer domains interact and are not independent in their contributions to enhancer activity. *J. Virol.* **63**, 3353-3361.

Holmes, B., Page, A.R. and Good, R.A. (1967) Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocyte function. *J Clin. Invest.* **46**, 1422-1432.

Hutt-Taylor, S.R., Harnish, D., Richardson, M., Ishizaka, T., and Denburg, J.A. (1988) sodium butyrate and a T cell line derived differentiation factor induce basophilic differentiation of the human promyelocytic cell line HL-60. *Blood* **71**, 209-215.

Im, D.S., and Muzyczka. (1989) Factors that bind to adeno-associated virus terminal repeats. *J. Virol.* **63**, 3095-3104.

Im, D.S., and Muzyczka. (1990) The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. *Cell* **61**, 447-457.

Im, D.S., and Muzyczka. (1992) Partial purification of adeno-associated virus Rep78, Rep68, Rep52, and Rep40 and their biochemical characterisation. *J. Virol.* **66**, 1119-1128.

Imperiale, M.J., Kao, H.-T., Feldman, L.T., Nevins, J.R., and Strickland, S. (1984) Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1A-like activity. *Mol. Cell Biol.* **4**, 867-874.

Issemann, I., and Green, S. (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* **347**, 645-650.

Janeway, C.A. and Gitlin, D. (1954) Hypergammaglobulinemia associated with severe, recurrent and chronic non-specific infection. *Am. J. Dis. Child* **88**, 388.

Jelinek, W.R., and Schmid, C.W. (1982) Repetitive sequences in eukaryotic DNA and their exression. *Ann. Rev. Biochem.* **51**, 813-844.

Jendrossek, V., Peters, A.M.J., Buth, S., Liese, J., Wintergerst, U., Belohradsky, B.H. and Gahr, M. (1993) Improvement of superoxide production in monocytes from patients with chronic granulomatous disease by recombinant cytokines. *Blood* **81**, 2131-2136.

Jolly, D. (1994) Viral vector systems for gene therapy. *Cancer Gene Ther.* **1**, 51-64.

Jones, N., and Shenk, T. (1979) Isolation of adenovirus type 5 deletion mutants defective for transformation of rat embryo cells. *Cell* **17**, 683-689.

Kakinuma, K., Kaneda, M., Chiba, T. and Ohnishi, T. (1986) Electron spin resonance studies on a flavoprotein in neutrophil plasma membranes. Redox potentials of the flavin and its participation in NADPH oxidase. *J. Biol. Chem.* **261**, 9426-9432.

Kamel-Reid, S., and Dick, J.E. (1988) Engraftment of immune-deficient mice with human hematopoietic stem cells. *Science* **242**, 1706-1709.

Karnovsky, M.L. and Badwey, J.A. (1986) Respiratory burst during phagocytosis: an overview. *Methods Enzymol.* **132**, 353-354.

Kariya, Y., Kato, K., Hayashizaki, Y., Himeno, S., Tarui, S., and Matsubara, K. (1987) Revision of consensus sequence of human Alu repeats-a review. *Gene* **53**, 1-10.

Katz, S., Kowenz-Leutz, E., Muller, C., Meese, K., Ness, S.A., and Leutz A. (1993) The NF-M trascription factor is related to C/EBP beta and plays a role in signal transduction, differentiation, and leukemogenesis of avian myelomonocytic cells. *EMBO J.* **12**, 1321-1332.

Kavanaugh, M.P., Miller, D.G., Zhang, W., Law, W., Kozak, S.L., Kabat, D., and Miller, A.D. (1994) Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-dependent phosphate symporters. *Proc. Natl. Acad. Sci. USA* **68**, 8270-8276.

Kay, H.E.M. (1965) How many cell generations? *Lancet* **2**, 418.

Keller, G. (1992) Hematopoietic stem cells. *Curr. Biol.* **4**, 133-139.

Kellum, R., and Schedl, P. (1991) A position-effect assay for boundaries of higher order chromosomal domains. *Cell* **64**, 941-950.

Kempler, G., Freitag, B., Berwin, B., Nanassy, O., and Barklis, E. (1993) Characterisation of the Moloney murine leukemia virus stem cell-specific repressor binding site. *Virology* **193**, 690-699.

Kenney, R.T., Malech, H.L., Gallin, J.I. and Leto, T.L. (1990) Amplification mapping of p67-*phox* deficient chronic granulomatous disease. *Clin. Res.* **38**, 434A.

Kenney, R.T., Malech, H.L., Roberts, R.L., Gallin, J. and Leto, T.L. (1991) Amniocentesis diagnosis of a normal foetus in a family with p67*phox* deficient chronic granulomatous disease using a polymorphic gene marker. *Clin. Res* **39**, 274A.

Kenney, R.T., Malech, H.L. and Leto, T.L. (1992) Structural characterization of the p67-*phox* gene. *Clin. Res.* **40**, 261A.

Kliewer, S.A., Umesono, K., Mangelsdorf, D.J., and Evans, R.M. (1992) Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D_3 signalling. *Nature* **355**, 446-449.

Klebanoff, S.J. (1988) Phagocytic cells: Products of Oxygen metabolism. In: *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J.I., Goldstein, I.M. and Snyderman, R. Eds.), New York: Raven Press, 391.

Klemsz, M.J., McKercher, S.R., Celada, C., Van Beveran, C., and Maki, R.A. (1990) The macrophage and B cell-specific transcription factor PU1 is related to the ets oncogene. *Cell* **61**, 113-24.

Kleinschmidt, J.A., Mohler, M., Weindler, F.W., and Heilbronn, R. (1995) Sequence elements of the adeno-associated virus rep gene required for suppression of herpes simplex virus-induced DNA amplification. *Virology* **206**, 254-262.

Knaus, U.G., Heyworth, P.G., Evans, T., Curnutte, J.T. and Bokoch, G.M. (1992) Regulation of phagocyte oxygen radical production by the GTP-binding protein rac2. *Science* **254**, 1512-1515.

Koeffler, H.P. (1985) In: *Chronic and acute leukemias in adults.* (Bloomfield C.D. Ed.) Boston, Martinus Nijhoff, 27.

Kotin, R.M., Siniscalco, M., Samulski, R.J., Zhu, X., Hunter, L., Laughlin, C.A., Mclaughlin, S., Muzyczka, N., Rocchi, M., Berns, K.I. (1990) Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA* **87**, 2211-2215.

Kotin, R.M., Menninger, J.C., Ward, D.C., Berns, K.I. (1991) Mapping and direct visualisation of a region specific viral DNA integration site on chromosome 19q13-qter. *Genomics* **10**, 831-834.

Kotin, R.M., Linden, R.M., Berns, K.I. (1992) Characterisation of a preferred site on human chromosome 19q for integration of adeno-associated virus by non-homologous recombination. *EMBO J.* **11**, 5071-5078.

Kotin, R.M. (1994) Prospects for the use of adeno-associated virus as a vector for human gene therapy. *Hum. Gen. Ther.* **5**, 793-801.

Kramer, I.M., Verhoeven, A.J., van der, B.R.L., Weening, R.S. and Roos, D. (1988) Purified protein kinase C phosphorylates a 47-kDa protein in control neutrophil cytoplasts but not in neutrophil cytoplasts from patients with the autosomal form of chronic granulomatous disease. *J. Biol. Chem.* **263**, 2352-2357.

Kramer, I.M., Van der Bend, R.L., Tool, A.T.J., Van Blitterswijk, W.J., Roos, D. and Verhoeven, A.J. (1989) 1-0-hexadecyl-2-0-methylglycerol, a novel inhibitor of PKC, inhibits the respiratory burst in human neutrophils. *J. Biol. Chem* **264**, 5876-5884.

Krieder, B.L., Benezra, R., Rovera, G., and Kadesch, T. (1992) Inhibition of myeloid differentiation by the helix-loop-helix protein Id. *Science* **255**, 1700-1702.

Kuff, E.L., and Lueders, K.K. (1988) The intracisternal A-particle gene family: structure and functional aspects. *Adv. Cancer Res.* **51**, 183-276.

LaMarco, K.L., and McKnight, S.L. (1989) Purification of a set of cellular polypeptides that bind to purine-rich cis-regulatory elements of herpes simplex virus immediate early genes. *Genes & Dev.* **3**, 1372-1383.

Landing, B.H. and Shirkey, H.S. (1957) A syndrome of recurrent infection and infiltration of viscera by pigmented lipid histiocytes. *Pediatr.* **20**, 431-438.

Lapidot, T., Pflumio, F., Doedens, M., Murdoch, B., Williams, D.E., and Dick, J.E. (1992) Cytokine stimulation of multilineage haematopoiesis from immature human cells engrafted in SCID mice. *Science* **255**, 1137-1141.

Leary, A.G., Strauss, L.C., Civin, C.I., and Ogawa, M. (1985) Disparate differentiation in hematopoietic colonies derived from human paired progenitors. *Blood* **66**, 327-332.

Lebkowski, J.S., McNally, M.M., Okarma, T.B., and Lerch, L.B. (1988) Adeno-associated virus: a vector system for efficient introduction of DNA into a variety of mammalian cell types. *Mol. Cell Biol.* **8**, 3988-3996.

Leonhardt, H., Page, A.W., Weier, H.-U., and Bestor, T.H. (1992) A targeting sequence directs DNA methyltransferases to sites of DNA replication in mammalian nuclei. *Cell* **71**, 865-873.

Leto, T.L., Lomax, K.J., Volpp, B.D., Nunoi, H., Sechler, J.M.G., Nauseef, W.M., Clark, R.A., Gallin, J.I. and Malech, H.L. (1990) Cloning of a 67-kD neutrophil oxidase factor with similarity to a noncatalytic region of $p60^{c\text{-}src}$. *Science* **248**, 727-730.

Leto,T.L., Adams, A.G., and de Mendez, I. (1994) Assembly of the phagocyte NADPH-oxidase: binding of Src homology 3 (SH3) domains to proline-rich targets. *Proc. Natl. Acad. Sci. USA*. **91**, 10650-10654.

Leusen, J.H.W., Bolscher, B.G.J.M., Hilarius, P.M., Weening, R.S., Kaulfersch, W., Seger, R.A., Roos, D., and Verhoeven, A.J. (1994) ^{156}Pro-Gln substitution in the light

chain of the human NADPH oxidase (p22-*phox*) leads to defective translocation of the cytosolic proteins p47-*phox* and p67-*phox*. *J. Exp. Med.* **180**, 2329-2334.

Levinsky, R.J., Harvey, B.A.M., Rodeck, C.H. and Soothill, J.F. (1983) Phorbol-myristate acetate stimulated NBT test; a simple method suitable for antenatal diagnosis of chronic granulomatous disease. *Clin. Exp. Immunol.* **54**, 595-598.

Levy, R., Rotrosen, D., Nagauker, O., Leto, T.L., and Malech, H.L. (1990) Induction of the respiratory burst in HL-60 cells. *J. Immunol.* **145**, 2595-2601.

Lewin, B. (1994) Chromatin and gene expression: constant questions, but changing answers. *Cell* **79**, 397-406.

Lewis, J., and Bird, A. (1991) DNA methylation and chromatin structure. *FEBS Lett.* **285**, 155-159.

Li, E., Bestor, T.H., and Jaenisch, R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69**, 915-926.

Li, F., Linton, G.F., Sekhsaria, S., Whiting-Theobold, N., Katkin, J.P., Gallin, J.I., and Malech, H.L. (1994) CD34+ peripheral blood progenitors as a target for genetic correction of the 2 flavocytochrome b_{558}-deficient forms of chronic granulomatous disease. *Blood* **84**, 53-58.

Li, J.-P., and Baltimore, D. (1991) Mechanism of leukemogenesis induced by mink cell focus-forming murine leukemia viruses. *J. Virol.* **65**, 2408-14.

Lord, K.A., Abdollahi, A., Hoffman-Lieberman, B., and Liebermann, D.A. (1993) Proto-oncogenes of the fos/jun family of transcription factors are positive regulators of myeloid differentiation. *Mol. Cell. Biol.* **13**, 841-851.

Lorenz, E., Congdon, C.C., Uphoff,D. (1952) Modification of acute radiation injury in mice and guinea pigs by bone marrow injections. *Radiology* **58**, 863-877.

Lowenstein, C.J. and Snyder, S.H. (1992) Nitric Oxide, a novel biologic messenger. *Cell* **70**, 705-707.

Luciw, P.A., and Leung, N.J. (1992) Mechanisms of retrovirus replication. In: *The Retroviridae, Volume 1,* (Levy, J.A. Ed.) Plenum Press, New York, 159-298.

Lutter, R., van Schaik, M.L., van Zwieten, R., Wever, R., Roos, D. and Hamers, M.N. (1985) Purification and partial characterization of the b-type cytochrome from human polymorphonuclear leukocytes. *J. Biol. Chem.* **260**, 2237-2244.

Mandel, M., and Higa, A. (1970) Calcium-dependent bacteriophage DNA infection. *J. Mol. Biol.* **53**, 159.

Mann, R., Mulligan, R., and Baltimore, D. (1983) Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33**, 153-159.

Maly, F.E., Cross, A.R., Jones, O.T., Wolf Vorbeck, G., Walker, C. and De Weck, A.L. (1988) The superoxide generating system of B cell lines. Structural homology with the phagocytic oxidase and triggering via surface Ig. *J Immunol.* **140**, 2334-2339.

Maly, F.E., Nakamura, M., Gauchat, A., Urwyler, C., Walker, C., Dahinden, C.A., Cross, A.R., Jones, O.T. and and DeWeck, A.L. (1990) Superoxide dependent nitroblue tetrazolium reduction and expression of cytochrome b_{-245} components by human B-lymphocytes and B-cell lines. *J. Immunol.* **142**, 1260-1267.

Maly, F.E., Schuerer-Maly, C.C., Quilliam, L., Cochrane, C.G., Newberger, P.E., Curnutte, J.T., Gifford, M., and Dinauer, M.C. (1993) Restitution of superoxide generation in autosomal cytochrome-negative chronic granulomatous disease B lymphocyte cell lines by transfection with p22*phox* cDNA. *J. Exp. Med.* **178**, 2047-2053.

Margolis, D.M., Melwick, D.A., Alling, D.W. and Gallin, J.I. (1990) Trimethoprim-Sulfamethoxazole prophylaxis in the management of chronic granulomatous disease. *J. Inf. Dis.* **162**, 723-726.

Maridonneau-Parini, I. and de Gunzburg, J. (1993) *J. Biol. Chem* **267**, 6395-6402.

Markowitz, D., Goff, S., and Bank, A. (1988) A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J. Virol.* **62**, 1120-1124.

McCarty, D.M., Christensen, M., and Muzyczka, N. (1991) Sequences required for the co-ordinate induction of the AAV p19 and p40 promoters by the Rep protein. *J. Virol.* **65**, 2936-2945.

McCarty, D.M., Pereira, D.J., Zolotukhin, I., Zhou, X., Ryan, J.H., and Muzyczka, N. (1994a) Identification of linear DNA sequences that specifically bind the adeno-associated virus Rep protein. *J. Virol.* **68**, 4988-4997.

McCarty, D.M., Ryan, J.H , Zolotukhin, S., Zhou, X., and Muzyczka, N. (1994b) Interaction of the adeno-associated virus Rep protein with a sequence within the A palindrome of the viral terminal repeat. *J. Virol.* **68**, 4998-5006.

McCune, J., Namikawa, R., Kaneshima, H., Schultz, L.D., Lieberman, M., and Weissman, I.L. The SCID-hu mouse: murine models for the analysis of human hematolymphoid differentiation and function. (1988) *Science* **241**, 1632-1639.

McGrory, W.J., Bautista, D.S., and Graham, F.L. (1988) A simple technique for the rescue of early region 1 mutants into infectious human adenovirus type 5. *Virology* **163**, 614-617.

McPhail, L.C., Shirley, P.S., Clayton, C.C. and Snyderman, R. (1985) Activation of the respiratory burst enzyme from human neutrophils in a cell-free system. *J Clin. Invest.* **75**, 1735-1739.

McPhail, L.C. SH3-dependent assembly of the phagocyte NADPH oxidase. *J. Exp. Med.* **180**, 2011-2015.

Metcalf, D. (1983) *Leukemia Res.* **7**,117.

Metcalf, D. (1991) Lineage commitment of hematopoietic progenitor cells in developing blast cell colonies: influence of colony-stimulating factors. *Proc. Natl. Acad. Sci. USA* **88**, 11310.

Michael, S.I., and Curiel, D.T. (1994) Strategies to achieve targeted gene delivery via the receptor-mediated endocytosis pathway. *Gene Therapy* **1**, 223-232.

Miller, A.D., Law, M.-F., and Verma, I.M. (1985) Generation of helper-free amphotropic retroviruses that transduce a dominant-acting, methotrexate-resistant dihydrofolate reductase gene. *Mol. Cell. Biol.* **5**, 431-437.

Miller, A.D., and Buttimore, C. (1986) Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell. Biol.* **6**, 2895-2902.

Miller, D.G., Edwards, R.H., and Miller, A.D. (1994) Cloning of the cellular receptor for amphotropic murine retroviruses reveals homology to that for gibbon ape leukemia virus. *Proc. Natl. Acad. Sci. USA* **91**, 78-82.

Miller, J.L., Donahue, R.E., Sellers, S.E., Samulski, R.J., Young, N.S., and Neinhuis, A.W. (1994) Recombinant adeno-associated virus mediated expression of a human γ-globin gene in human progenitor-derived erythroid cells. *Proc. Natl. Acad. Sci. USA* 91, 10183-10187.

Mitani, K., Graham, F.L., and Caskey, C.T. (1994) Transduction of human bone marrow by adenoviral vector. *Hum. Gene Ther.* **5**, 941-948.

Moll, J., Sansig, G., Fattori, E. and Van der Putten, H. The murine rac1 gene: cDNA cloning, tissue distribution and regulated expression of rac1 mRNA by dissassembly of actin microfilaments. (1991) *Oncogene* **6**, 863-866.

Momoeda, M., Kawase, M., Jane, S.M., Miyamura, K., Young, N.S., and Kajigaya, S. (1994) The transcriptional regulator YY1 binds to the 5'-terminal region of B19 parvovirus and regulates p6 promoter activity. *J. Virol.* **68**, 7159-7168.

Moore, M.A.S. (1991) Clinical implications of positive and negative hematopoietic stem cell regulators. *Blood* **78**, 1-19.

Morel, F., Doussiere, J. and Vignais, P.V. (1991) The superoxide-generating oxidase of phagocytic cells. Physiological, molecular and pathological aspects. *Eur. J. Biochem.* **201**, 523-546.

Morgan, G. (1992) Bone marrow transplantation for immunodeficiency syndromes. In: *Bone marrow transplantation in practice* (Treleaven, J. and Barrett, J. Eds) Churchill Livingstone, 119-135.

Morgan, R.A., Nussbaum, O., Muenchau, D.D., Shu, L., Couture, L., and French Anderson, F. (1993) Analysis of the functional and host-range determining regions of the murine ecotropic and amphotropic envelope proteins. *J. Virol* **67**, 4712-4721.

Morgenstern, J.P., and Land, H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* **18**, 3587-3596.

Mouy, R., Fischer, A., Vilmer, E., Seger, R. and Griscelli, C. (1989) Incidence, severity and prevention of infections in chronic granulomatous disease. *J. Pediatr.* **114**, 555-560.

Mouy, R., Seger, R., Bourquin, J.P., Veber, F., Blanche, S., Griscelli, C. and Fischer, A. (1991) Interferon gamma for chronic granulomatous disease. *N. Engl. J. Med.* **325**, 1516.

Muhlebach, T.J., Robinson, W., Seger, R.A. and Machler, M. (1990) A Second Nsi1 RFLP at the CYBB Locus. *Nucleic Acids Res.* **18**, 4966.

Muhlebach, T.J., Feickert, H.J., Welte, K. and Seger, R.A. (1991) Granulocyte-macrophage colony stimulating factor does not improve neutrophil oxidative metabolism in a patient with X-linked variant chronic granulomatous disease. *Eur. J. Pediatr* **8**, 575-578.

Muhlebach, T.J., Gabay, J., Nathan, C.F., Erny, C., Dopfer, G., Schroten, H., Wahn, V. and Seger, R.A. (1992) Treatment of patients with chronic granulomatous disease with recombinant human γ-interferon does not improve neutrophil oxidative metabolism, cytochrome b_{558} content or levels of four antimicrobial proteins. *Clin. Exp. Immunol.* **88**, 203-206.

Mulligan, R.C., and Berg, P. Selection of animal cells that express the Escherichia coli gene for xanthine-guanine phosphoribosyl transferase (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2072-2076.

Murray, L., Chen, B., Galy, A., Chen, S., Tushinski, R., Uchida, N., Negrin, R., Tricot, G., Jagannath, S., Vesole, D., et al. (1995) Enrichment of human hematopoietic stem cell activity in the CD34+Thy-1+Lin- subpopulation from mobilised peripheral blood. *Blood* **85**, 368-378.

Musacchio, A., Gibson, T., Lehto, V. and Saraste, M. (1992) SH3-an abundant protein domain in search of a function. *FEBS* **307**, 55-61.

Muzyczka,N. (1992) Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr. Top. Microbiol. Immunol.* **158**, 97-129.

Naar, A.M., Boutin, J., Lipkin, S.M., Holloway, J.M., Glass, C.M. and Rosenfeld, M.G. (1991) The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. *Cell* **65**, 1267-1279.

Nahreini, P., Larsen, S.H., and Srivistava, A. (1992) Cloning and integration of DNA fragments in human cells via the inverted terminal repeats of the adeno-associated virus 2 genome. *Gene* **119**, 265-272.

Nakahata, T., Gross, A.J., and Ogawa, M. (1982) A stochastic model of self-renewal and commitment to differentiation of the primitive hematopoietic stem cells in culture. *J. Cell Physiol.* **113**, 455.

Nakamura, M., Imajoh-Ohmi, S., Kanegesaki, S., Kurozumi, H., Sato, K., Kato, S. and Miyazaki, Y. (1990) Prenatal diagnosis of cytochrome-deficient chronic granulomatous disease. *Lancet* **336**, 118-119.

Natesan, S., and Gilman, M.Z. (1993) DNA-bending and orientation-dependent fumction of YY1 in the c-fos promoter. *Genes Dev.* **7**, 2497-2509.

Nauseef, W.M., Volpp, B.D., McCormick, S., Leidal, K.G. and Clark, R.A. (1991) Assembly of the neutrophil respiratory burst oxidase. *J. Biol. Chem.* **266**, 5911-5917.

Newburger, P.E., Cohen, H.J., Rothchild, S.B., Hobbins, J.C., Malawista, S.E. and Mahoney, M.J. (1979) Prenatal diagnosis of chronic granulomatous disease. *N. Engl. J. Med.* **300**, 178-181.

Newberger, P.E., Ezekowitz, R.A.B., Whitney, C., Wright, J., and Orkin, S.H. (1988) Induction of phagocyte cytochrome b heavy chain gene expression by IFN-γ. *Proc. Natl. Acad. Sci. USA* **85**, 5215-5219.

Newburger, P.E., Skalnik, D.J., Hopkins, P.J., Eklund, E.A., and Curnutte, J.T. (1994) Mutations in the promoter region of the gene for gp91-*phox* in X-linked chronic granulomatous disease with decreased expression of cytochrome b_{558}. *J. Clin. Invest.* **3**, 1205-1211.

Nunoi, H., Rotrosen, D., Gallin, J.I. and Malech, H.L. (1988) Two forms of autosomal chronic granulomatous disease lack distinct neutrophil cytosol factors. *Science* **242**, 1298-1301.

O'Hara, B., Johann, H.P., Klinger, H.P., Blair, D.G., Rubinson, H., Dunn, K.J., Sass, P., Vitek, S.M., and Robins, T. (1990) Characterisation of a human gene conferring sensitivity to infection by gibbon ape leukemia virus. *Cell Growth Differ.* **1**, 119-127.

Ochs, H.D. and Igo, R.P. (1973) The NBT slide test: A simple screening method for detecting CGD and female carriers. *J. Paediatr* **83**, 77.

Odell, E.W. and Segal, A.W. (1988) The bactericidal effects of the respiratory burst and the myeloperoxidase system isolated in neutrophil cytoplasts. *Biochim. Biophys. Acta* **971**, 266-274.

Odell, E.W. and Segal, A.W. (1991) Killing of pathogens associated with chronic granulomatous disease by the non-oxidative microbicidal mechanisms of human neutrophils. *J. Med. Microbiol* **34**, 129-135.

Ogawa, M. (1993) Differentiation and proliferation of hematopoietic stem cells. *Blood* **81**, 2844-2853.

Ohno, Y., Buescher, E.S., Roberts, R., Metcalf, J.A. and Gallin, J.I. (1986) Reevaluation of cytochrome b and flavin adenine dinucleotide in neutrophils from patients with chronic granulomatous disease and description of a family with probable autosomal recessive inheritance of cytochrome b deficiency. *Blood* **67**, 1132-1138.

Ohta, H., Takahashi, H., Hattori, H., Yamada, H. and Takikawa, K. (1966) Some oxidative enzymes and cytochrome in the specific granules of neutrophile leukocytes. *Acta Haematol Jap* **29**, 799-808.

Okamura, N., Curnutte, J.T., Roberts, R.L. and Babior, B.M. (1988) Relationship of protein phosphorylation to the activation of the respiratory burst in human neutrophils. Defects in the phosphorylation of a group of closely related 48-kDa proteins in two forms of chronic granulomatous disease. *J. Biol. Chem.* **263**, 6777-6782.

Pahl, H.L., Schiebe, R.J., Zang, D.E., Chen, H., Galson, D.L., Maki, R.A. and Tenen, D.G. (1993) The proto-oncongene PU.1 regulates expression of the myeloid-specific CD11b promoter. *J. Biol. Chem.* **267**, 5014-5020.

Parker, M.G. (1993) Steroid and related receptors. *Curr. Opin. Cell Biol.* **5**, 499-504.

Parkos, C.A., Allen, R.A., Cochrane, C.G. and Jesaitis, A.J. (1987) Purified cytochrome b from human granulocyte plasma membrane is comprised of two polypeptides with relative molecular weights of 91,000 and 22,000. *J Clin. Invest.* **80**, 732-742.

Parkos, C.A., Dinauer, M.C., Walker, L.E., Allen, R.A., Jesaitis, A.J. and Orkin, S.H. (1988) Primary structure and unique expression of the 22-kilodalton light chain of human neutrophil cytochrome b. *Proc. Natl. Acad. Sci. USA* **85**, 3319-3323.

Parkos, C.A., Dinauer, M.C., Jesaitis, A.J., Orkin, S.H. and Curnutte, J.T. (1989) Absence of both the 91kD and 22kD subunits of human neutrophil cytochrome b in two genetic forms of chronic granulomatous disease. *Blood* **73**, 1416-1420.

Pelham, A., O'Reilly, M.J., Malcolm, S., Levinsky, R.J. and Kinnon, C. (1990) RFLP and deletion analysis for X-linked chronic granulomatous disease using the cDNA probe: potential for improved prenatal diagnosis and carrier demonstration. *Blood* **76**, 820-824.

Pember, S.O., Heyl, B.L., Kinkade, J.M.J. and Lambeth, J.D. (1984) Cytochrome b_{558} from (bovine) granulocytes. Partial purification from Triton X-114 extracts and properties of the isolated cytochrome. *J. Biol. Chem.* **259**, 10590-10595.

Perez, C., Coeffier, E., Moreau-Gachelin, F., Wietzerbin, J. and Benech, P.D. (1994) Involvement of the transcription factor PU.1/Spi-1 in myeloid cell-restricted expression of an interferon-inducible gene encoding the human high-affinity Fcg receptor. *Mol. Cell. Biol.* **14**, 5023-5031.

Pfeifer, G.P., Steigerwald, S.D., Hansen, R.S., Gartler, S.M., and Riggs, A.D. (1990) Polymerase chain reaction-aided genomic sequencing of an X chromosome-linked CpG island: methylation patterns suggest clonal inheritance, CpG site autonomy, and an explanation of active state stability. *Proc. Natl. Acad. Sci. USA* **87**, 8252-8256.

Pfeifer, J.D., Wick, M.J., Roberts, R.L., Findlay, K., Normark, S.J. and Harding, C.V. (1993) Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* **361**, 359-362.

Philip, R., Brunette, E., Kilinski, L., Murugesh, D., McNally, M.M., Ucar, K., Rosenblatt, J., Okarma, T.B., and Lebkowski, J.S. (1994) Efficient and sustained gene expression in primary T lymphocytes and primary and cultured tumor cells mediated by adeno-associated virus plasmid DNA complexed to cationic liposomes. *Mol. Cell Biol.* **14**, 2411-2418.

Pick, E., Kroizman, T. and Abo, A. (1989) Activation of the superoxide-forming NADPH oxidase of macrophages requires two cytosolic components-one of them is also present in certain non-phagocytic cells. *J. Immunol.* **143**, 4180-4187.

Pollock, J.D., Williams, D.A., Gifford, M.A.C., Li, L.L., Du, X., Fisherman, J., Orkin, S.H., Doerschuk, C.M., and Dinauer, M.C. (1995) Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nature Genetics.*. 9, 202-209.

Porter, C.D., Parkar, M.H., Collins, M.K.L., Levinsky, R.J. and Kinnon, C. (1992) Superoxide production measured by chemiluminescence-based assays by normal and chronic granulomatous disease (CGD) patient-derived EBV-transformed B cell Lines. *J. Immunol. Meth.* **155**, 151-157.

Porter, C.D., Parkar, M.H., Levinsky, R.J., Collins, M.L.K., and Kinnon, C. (1993) X-linked chronic granulomatous disease; correction of NADPH-oxidase defect by retrovirus mediated gene transfer. *Blood* **82**, 2196-2202.

Porter, C.D., Parkar, M.H., Verhoeven, A.J., Levinsky, R.J., Collins, M.K.L., and Kinnon, C. (1994) p22-*phox* deficient chronic granulomatous disease: reconstitution by retrovirus-mediated expression and identification of a biosynthetic intermediate of gp91-*phox*. *Blood* **84**, 2767-2775.

Porter, T.D. (1991) An unusual yet strongly conserved flavoprotein reductase in bacteria and mammals. *TIBS* **16**, 154-158.

Poynton, C. (1992) T cell depletion in bone marrow transplantation. In: *Bone marrow transplantation in practice* (Treleaven, J. and Barrett, J. Eds) Churchill Livingstone, 227-237.

Qu, A.Y., Nanda, A., Curnutte, J.T., and Grinstein, S. (1994) Development of a H+-conductance during granulocytic differentiation of HL60 cells. *Am. J. Physiol.* **266** (5 pt 1), C1263-1270.

Quie, P.G., White, J.G., Holmes, B. and Good, R.A. (1967) In vitro bactericidal capacity of human polymorphonuclear leukocytes: diminished activity in chronic granulomatous disease of childhood. *J Clin. Invest.* **46**, 668-679.

Quie, P.G. (1993) Chronic granulomatous disease of childhood: a saga of discovery and understanding. *Pediatr. Infect. Dis. J.* **12**, 395-398.

Quilliam, L.A., Mueller, H., Bohl, B.P., Prossnitz, V., Sklar, L.A. and Der, C.J. (1991) Rap1A is a substrate for cyclic AMP-dependent protein kinase in human neutrophils *J. Immunol* **147**, 1628-1635.

Quinn, M.T., Parkos, C.A., Walker, L., Orkin, S.H., Dinauer, M.C. and Jesaitis, A.J. (1989) Association of a Ras-related protein with cytochrome b of human neutrophils. *Nature* **342**, 198-200.

Quinn, M.T., Mullen, L.M. and Jesaitis, A.J. (1992) Human neutrophil cytochrome b contains multiple hemes. *J. Biol. Chem.* **267**, 7303-7309.

Quinn, M.T., Mullen, M.L., Jesaitis, A.J. and Linner, D.G. (1993) *Blood* **79**, 1563-1573.

Radeke, H.H., Cross, A.R., Hancock, J.T., Jones, O.T.G., Nakamura, M., Kaever, V. and Resch, K. (1991) Functional expression of NADPH oxidase components (alpha and beta subunits of cytochrome b_{558} and 45-kDa flavoprotein) by intrinsic human glomerular mesangial cells. *J. Biol. Chem.* **266**, 21025-21029.

Razin, A., and Riggs, A.D. (1980) DNA methylation and gene function. *Science* **210**, 604-610.

Reeves, R. (1984) Transcriptionally active chromatin. *Biochim. Biophys. Acta.* **782**, 343-393.

Reibel, L., Dorseuil, O., Stancou, R., Bertoglio, J. and Gacon, G. (1991) A hemopoietic-specific gene encoding a small GTP-binding protein is over-expressed during T cell activation. *Biochem. Biophys. Res. Commun* **175**, 451-458.

Reisman, D., and Rotter, V. (1989) Induced expression from the Moloney murine leukemia virus long terminal repeat during differentiation of human myeloid cells is mediated through its transcriptional enhancer. *Mol. Cell. Biol.* **9**, 3571-3575.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D. and Hall, A. (1992) The small GTP binding protein rac regulates growth factor induced membrane ruffling. *Cell* **70**, 401-410.

Roberts, P.J., Cross, A.R., Jones, O.T. and Segal, A.W. (1982) Development of cytochrome b and an active oxidase system in association with maturation of a human promyelocytic (HL-60) cell line. *J Cell. Biol.* **95**, 720-726.

Roberts, P.J., Jones, M., Gale, R., Thomas, S., Tidman, N., and Linch, D. (1989) The *c-myc* oncogene is regulated independently of differentiation in myeloid cells. *Leuk. Res.* **13**, 651-9.

Rodaway, A.R.F., Teahan, C.G., Casimir, C.M., Segal, A.W. and Bentley, D.L. (1990) Characterisation of the 47kD autosomal chronic granulomatous disease protein; tissue specific expression and transcriptional control by retinoic acid. *Mol. Cell. Biol.* **10**, 5388-5396.

Roe, T., Reynolds, T.C., Yu, G., and Brown, P.O. (1993) Integration of murine leukemia virus DNA depends on mitosis. *EMBO J.* **12**, 2099-2108.

Rogers, J. (1985) Oncogene chromosome breakpoints and Alu sequences. *Nature* **317**, 559.

Roos, D., de Boer, M., Borregaard, N., Bjerrum, O.W., Valerius, N.H., Seger, R.A., Muhlebach, T., Belhohradsky, B.H. and Weening, R.S. (1992) Chronic granulomatous disease with partial deficiency of cytochrome b_{558} and incomplete respiratory burst: variants of the X-linked, cytochrome b_{558} negative form of the disease. *J. Leukocyte. Biol* **51**, 164-171.

Roos, D., de Boer, M., De Klein, A., Bolscher, B.G.J.M. and Weening, R.S. (1993) Chronic granulomatous disease: mutations in cytochrome b_{558}. *Immunodeficiency* **4**, 289-302.

Rossi, F., and Zatti, M. (1964) Biochemical aspects of phagocytosis in polymorphonuclear leukocytes. NADH and NADPH oxidation by the granules of resting and phagocytosing cells. *Experimentia* **20**, 21.

Rossi, F. (1986) The O_2^--forming NADPH oxidase of the phagocytes: nature, mechanisms of activation and function. *Biochim. Biophys. Acta* **853**, 65-89.

Rotrosen, D., Yeung, C.L., Leto, T.L., Malech, H.L. and Kwong, C.H. (1992a) Cytochrome b_{558}: the Flavin-Binding Component of the Phagocyte NADPH Oxidase. *Science* **256**, 1459-1462.

Rotrosen, D., Yeung, C.L., Leto, T.L., Malech, H.L. and Kwong, C.H. (1992b) Analysis of NADPH oxidase activation in a cell-free system comprised of purified protein components. *Clin. Res.* **40**, 351A.

Rotrosen, D., Yeung, C.L. and Katkin, J.P. (1993) Production of recombinant cytochrome b_{558} allows reconstitution of the phagocyte NADPH-oxidase solely from recombinant proteins. *J. Biol. Chem* **268**, 14256-14260.

Royer Pokora, B., Kunkel, L.M., Monaco, A.P., Goff, S.C., Newburger, P.E., Baehner, R.L., Cole, F.S., Curnutte, J.T. and Orkin, S.H. (1986) Cloning the gene for an inherited human disorder -chronic granulomatous disease- on the basis of its chromosomal location. *Nature* **322**, 32-38.

Russell, D.W., Miller, A.D., and Alexander, I.E. (1994) Adeno-associated virus vectors preferentially transduce cells in S phase. *Proc. Natl. Acad. Sci. USA* **91**, 8915-8919.

Sakai, T., Toguchida, J., Ohtani, N., Yandell, D.W., Rapaport, F.M., and Dryja, T.P. (1991) Allele specific hypermethylation of the retinoblastoma tumor-suppressor gene. *Am. J. Hum. Genet.* **48**, 880-888.

Samulski, R.J., Berns, K.I., Tan, M., and Muzyczka, N. (1982) Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. *Proc Natl. Acad. Sci. USA* **79**, 2077-2081.

Samulski, R.J., Chang, L-S., and Shenk, T. (1989) Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. *J. Virol.* **63**, 3822-3828.

Samulski, R.J., Zhu, X., Xiao, X., Brook, J.D., Housman, D.E., Epstein, N., and Hunter,, L.A. (1991) Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J.* **10**, 3941-3950.

Sbarra, A.J. and Karnovsky, M.L. (1959) The biochemical basis of phagocytosis: metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. *J. Biol. Chem.* **234**, 1355-1362.

Schapiro, B.L., Newburger, P.E., Klempner, M.S. and Dinauer, M.C. (1991) Chronic granulomatous disease presenting in a 69 year old man. *N. Engl. J. Med.* **325**, 1786-1790.

Schulz, L.D., Schweitzer, P.A., Christianson, S.W., Gott, B., Schweitzer, I.B., Tennent, B., McKenna, S., Mobraaten, L., Rajan, T.V., Greiner, D.L., and Leiter, E.H. (1995) Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J.Immunol.* **154**, 180-191.

Scott, E.W., Simon, M.C., Anastasi, J., and Singh, H. (1994) Requirement of transcription factor PU1 in the development of multiple haematopoietic lineages. *Science* **265**, 1573-1577.

Scott, L.M., Civin, C.I., Rorth, P., and Friedman, A.D. (1992) A novel temporal expression pattern of 3 C/EBP family members in differentiating myelomonocytic cells. *Blood* **80**, 1725-1735.

Segal, A.W. (1974) Nitroblue-tetrazolium tests. *Lancet* **2**, 1248-1252.

Segal, A.W., Geisow, M., Garcia, R., Harper, A. and Miller, R. (1981) The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH. *Nature* **290**, 406-409.

Segal, A.W., Cross, A.R., Garcia, R.C., Borregaard, N., Valerius, N.H., Soothill, J.F. and Jones, O.T. (1983) Absence of cytochrome b_{-245} in chronic granulomatous disease. A multicenter European evaluation of its incidence and relevance. *N. Engl. J Med.* **308**, 245-251.

Segal, A.W., Heyworth, P.G., Cockcroft, S. and Barrowman, M.M. (1985) Stimulated neutrophils from patients with autosomal recessive chronic granulomatous disease fail to phosphorylate a Mr-44,000 protein. *Nature* **316**, 547-549.

Segal, A.W. (1987) Absence of both cytochrome b_{-245} subunits from neutrophils in X- linked chronic granulomatous disease. *Nature* **326**, 88-91.

Segal, A.W., West, I., Wientjes, F.B., Nugent, J.H.A., Chavan, A.J., Haley, B., Garcia, R.C., Rosen, H. and Scrace, G. (1992) Cytochrome b_{-245} is a flavocytochrome containing FAD and the NADPH-binding site of the microbicidal oxidase of phagocytes. *Biochem. J.* **284**, 781-788.

Segal, A.W. and Abo, A. (1993) The biochemical basis of the NADPH oxidase of phagocytes. *TIBS* **18**, 43-47.

Segal, A.W. and Jones, O.T.G. (1978) Novel cytochrome b system in phagocytic vacuoles from human granulocytes. *Nature* **276**, 515-517.

Segal, A.W. and Jones, O.T.G. (1980) Absence of cytochrome b reduction in stimulated neutrophils from both female and male patients with chronic granulomatous disease. *FEBS. Lett.* **110**, 111-114.

Segal, A.W. and Peters, T.J. (1976) Characterisation of the enzyme defect in chronic granulomatous disease. *Lancet* **1**, 1363-1365.

Seger, R.A., and Ezekowitz, R.A.B. (1994) Treatment of chronic granulomatous disease. *Immunodeficiency* **5**, 113-130.

Sekhsaria, S., Gallin, J.I., Linton, G.F., Mallory, R.M., Mulligan, R.C., and Malech, H.L. (1993) Peripheral blood progenitors as a target for genetic correction of p47*phox*-deficient chronic granulomatous disease. *Proc. Natl. Acad. Sci. USA* **90**, 7446-7450.

Selsted, M., Miller, C.W., Novotny, M.J., Morris, W.L. and Koeffler, H.P. (1993) Molecular analysis of myeloperoxidase deficiency shows heterogeneous patterns of the complete deficiency state manifested at the genomic, mRNA, and protein levels. *Blood* **82**, 1317-1322.

Sengelv, H., Nielson, M.H., Borregaard, N. (1992) Separation of human neutrophil plasma membrane from intracellular vesicles containing alkaline phosphatase and NADPH oxidase activity by free flow electrophoresis. *J. Biol. Chem.* **267**, 14912-14917.

Serra, M.C., Bellavite, P., Davoli, A., Bannister, J.V. and Rossi, F. (1984) Isolation from neutrophil membranes of a complex containing active NADPH oxidase and cytochrome b_{-245}. *Biochim. Biophys. Acta* **788**, 138-146.

Seto, E., Shi, Y., and Shenk, T. (1991) YY1 is an initiator sequence-binding protein that directs and activates transcription in vitro. *Nature* **354**, 241-245.

Seto, E., Lewis, B., and Shenk, T. (1993) Interaction between transcription factors Sp1 and YY1. *Nature* **365**, 462-464.

Shelley, C.S., Farokhzad, O.C., and Arnaout, M.A. (1993) Identification of cell-specific and developmentally regulated nuclear factors that direct myeloid and lymphoid expression of the CD11a. *Proc. Natl. Acad. Sci. USA* **90**, 5364-5368.

Shi, Y., Seto, E., Chang, L-S., and Shenk, T. (1991) Transcriptional repression by YY1, a human GLI-Kruppel-related protein, and relief of repression by adenovirus E1A protein. *Cell* **67**, 377-388.

Shinagawa, Y., Tanaka, C. and Teraoka, A. (1966) A new cytochrome in neutrophilic granules of rabbit leucocytes. *J. Biochem (Tokyo)* **59**, 622-624.

Simmons, D.L., Satterthwaite, A.B., Tenen, D.G., and Seed, B. (1992) Molecular cloning of a cDNA encoding CD34, a sialomucin of human haematopoietic stem cells. *J. Immunol.* **148**, 267-71.

Skalnik, D.G., Strauss, E.C., and Orkin, S.H. (1991a) CCAAT displacement protein as a repressor of the myelomonocytic-specific gp91-*phox* gene promoter. *J. Biol. Chem.* **266**, 16736-16744.

Skalnik, D.G., Dorfman, D.M., Perkins, A.S., Jenkins, N.A., Copeland, N.G. and Orkin, S.H. (991b) Targeting of transgene expression to monocyte/macrophages by the gp91-*phox* promoter and consequent histiocytic malignancies. *Proc. Natl. Acad. Sci. USA* **88**, 8505-8509.

Slater, E.P., Redeuilh,G., and Beato, M. (1991) Hormonal regulation of vitellogenin genes: an estrogen responsive element in the Xenopus A2 gene and a multihormonal regulatory region in the chicken II gene. *Mol. Endocrinol.* **5**, 386-396.

Smale, S.T., and Baltimore, D. (1989) The 'initiator' as a transcription control element. *Cell* **57**, 103-113.

Snyder, R.O., Samulski, R.J., and Muzyczka, N. (1990) In vitro resolution of covalently joined AAV chromosome ends. *Cell* **60**, 105-113.

Someya, A., Nagaoka, I. and Yamashita, T. (1993) Purification of the 260 kDa complex involved in the superoxide production of guinea pig neutrophils. *FEBS. Lett* **330**, 215-218.

Speck, N., and Baltimore, D. (1987) Six distinct factors interact with the 75 base-pair repeat of the Moloney murine leukemia virus enhancer. *Mol. Cell. Biol.* **7**, 1101-1110.

Sprangrude, G.J., Heimfeld, S., and Weissman, I.L. (1988) Purification and characterisation of mouse haematopoietic stem cells. *Science* **241**, 58-62.

Steck, F.T., and Rubin, H. (1994) *Virology* **91**, 1168-1172.

Stewart, C., Stuhlman, H., Jahner, D., and Jaenisch, R. (1982) De novo methylation, expression, and infectivity of retroviral genomes introduced into embryonal carcinoma cells. *Proc. Natl. Acad. Sci. USA* **79**, 4098-4092.

Stewart, M.F., Crittenden, R.B.,Lowry, P.A., Pearson-White, S., and Qeensbury, P.J. (1993) Long term engraftment of normal and post 5-FU murine marrow into normal non-myeloablated mice. *Blood* **81**, 2566-2571.

Sturgill-Koszycki, S., Schlesinger, P.H., Chakrabarty, P., Haddix, P.L., Collins, H.L., Fok, A.K., Allen, R.D., Gluck, S.L., Heuser, J., and Russell, D.G. (1994) Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science* **263**, 678.

Suda, T., Suda, J., and Ogawa, M. (1983) Single cell origin of mouse haematopoietic colonies expressing multiple lineages in variable combinations. *Proc. Natl. Acad. Sci. USA* **80**, 6689.

Sumimoto, H., Sakamoto, N., Nozaki, M., Sakaki, Y., Takeshige, K. and Minakami, S. (1992) Cytochrome b_{558}, a component of the phagocyte NADPH oxidase, is a flavoprotein. *Biochem. Biophys. Res. Comm.* **186**, 1368-1375.

Sutcliffe, J.S., Nelson, D.L., Zhang, F., Pierretti, M., Caskey, S.T., Saxe, D., and Warren, S.T. (1992) DNA methylation represses FMR-1 transcription in fragile X syndrome. *Hum. Mol. Genet.* **1**, 397-400.

Sutherland, H.J., Eaves, C.J., Eaves, A.C., Dragowska, W., and Lansdorp, P.M. (1989) Characterisation and partial purification of human marrow cells capable of initiating long term hematopoiesis in vitro. *Blood* **74**, 1563-1570.

Swain, J.L., Stewart, T.A., and Leder, P. (1987) Parental legacy determines methylation and expression of an autosomal transgene: a mechanism for parental imprinting. *Cell* **50**, 719-727.

Tabin, C.J., Hoffman, J.W., Goff, S.P., and Weinberg, R.A. (1982) Adaption of a retrovirus as a eukaryotic vector in transmitting the herpes simplex virus thymidine kinase gene. *Mol. Cell Biol.* **2**,426.

Taylor, W.R., Jones, D.T. and Segal, A.W. (1993) A structural model for the nucleotide binding domain of cytochrome b_{-245} b-chain. *Prot. Sci.* **2**, 1675-1685.

Teahan, C., Rowe, P., Parker, P., Totty, N. and Segal, A.W. (1987) The X-linked chronic granulomatous disease gene codes for the beta-chain of cytochrome b_{-245}. *Nature* **327**, 720-721.

Thrasher, A.J., Chetty, M., Casimir, C.M., and Segal, A.W. (1992) Restoration of superoxide generation to a chronic granulomatous disease derived B-cell line by retrovirus mediated gene transfer. *Blood* **80**, 1125-1129.

Till, J.E., and McCulloch, E.A. (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* **14**, 213.

Till, J.E., McCulloch, E.A., and Siminovitch, L. (1964) A stochastic model of stem cell proliferation based on the growth of spleen colony-forming cells. *Proc. Natl. Acad. Sci. USA* **51**, 29.

Todd, P.A. and Goa, K.L. (1992) Interferon Gamma-1b: a review of its pharmacology and therapeutic potetial in chronic granulomatous disease. *Drugs* **43(1)**, 111-122.

Tratschin, J.D., Miller, I.L., and Carter, B.J. (1984) Genetic analysis of adeno-associated virus: properties of deletion mutants constructed in vitro and evidence for an adeno-associated virus replication function. *J. Virol.* **51**, 611-619.

Tratschin, J.D., Miller, I.L., Smith, M.G., and Carter, B.J. (1985) Adeno-associated virus vector for high frequency integration, expression and rescue of genes in mammalian cells. *Mol. Cell Biol.* **5**, 3251-3260.

Umesono, K., Murakami, K.K., Thompson, C.C. and Evans, R.M. (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3. *Cell* **65**, 1255-1266.

Uriel-Shoval, S., Grunebaum, Y., Sedat, J., and Razin, A. (1982) The absence of detectable methylated bases in *Drosophila melanogaster* DNA. *FEBS Lett.* **146**, 148-152.

Van Beusechem, V.W., Kukler, A., Einerhand, M.P.W., et al. (1990) Expression of human adenosine deaminase in mice transplanted with hematopoietic stem cells infected with amphotropic retroviruses. *J. Exp. Med.* **172**, 729-736.

Van Beusechem, V.W., Kukler, A., Heidt, P.J., and Valerio, D. (1992) Long-term expression of human adenosine deaminase in rhesus monkeys transplanted with retrovirus-infected bone marrow cells. *Proc. Natl. Acad. Sci. USA* **89**, 7640-7644.

Van Buesechem, V.W., Bakx, T.A., Kaptein, L.C.M. et al (1993) Retrovirus mediated gene transfer into rhesus monkey haematopoietic stem cells: the effect of viral titres on transduction efficiency. *Hum. Gene Ther.* **4**, 239-247.

van Zeijl, M., Johann, S.V., Closs, E., Cunningham, J., Shows, T.B., and O'Hara, B. (1994) A human amphotropic retrovirus receptor is a second member of the gibbon ape leukemia virus receptor family. *Proc. Natl. Acad. Sci. USA* **91**, 1168-1172.

van't Wout, J.M. (1992) Itraconazole in neutropenic patients. *Chemotherapy* **38** S1, 23-26.

Verhoeven, A.J., Van Schaik, M.L.J., Roos, D., and Weening, R.S. (1988) Detection of carriers of the autosomal form chronic granulomatous disease. *Blood* **71**, 505.

Visser, J.W.M. (1992) Isolation and characterisation of bone marrow stem cells and progenitor cells by flow cytometry. In: *Flow cytometry in hematology,* (Laerum, O.D., and Bjerknes, R. Eds.).

Volkman, D.J., Buescher, E.S., Gallin, J.I., and Fauci, A.S. (1984) B cell lines as models for inherited phagocytic diseases: abnormal superoxide generation in chronic granulomatous disease and giant granules in Chediak-Higashi syndrome. *J. Immunol.* **133**, 3006-3009.

Volkman, D.J.,Volpp, B.D., Nauseef, W.M. and Clark, R.A. (1988) Two cytosolic neutrophil oxidase components absent in autosomal chronic granulomatous disease. *Science* **242**, 1295-1297.

Volpp, B.D., Nauseef, W.M., Donelson, J.E., Moser, D.R. and Clark, R.A. (1989) Cloning of the cDNA and functional expression of the 47-kilodalton cytosolic component of human neutrophil respiratory burst oxidase. *Proc. Natl. Acad. Sci. USA* **86**, 7195-7199.

Volpp, B.D., and Lin, Y. (1993) In vitro molecular reconstitution of the respiratory burst in B-lymphoblasts from p47*phox* deficient chronic granulomatous disease. *J.Clin.Invest* **91**:201-207.

von Kalle, C., Kiem, H-P., Goehle, S., Darovsky, B., Heimfeld, S., Torok-Storb, B., Storb, R., and Schuening, F.G. (1994) Increased gene transfer into human haematopoietic progenitor cells by extended in vitro exposure to a pseudotyped retroviral vector. *Blood* **84**, 2890-2897.

Walsh, C.E., Neinhuis, A.W., Samulski, R.J., Brown, M.G., Miller, J.L., Young, N.S., and Liu, J.M. (1994) Phenotypic correction of Fanconi anemia in human hematopoietic cells with a recombinant adeno-associated virus vector. *J. Clin. Invest.* **94**, 1440-1448.

Wang, L.H., Ing, N.H., Tsai, S.Y., O'Malley, B.W. and Tsai, M.J. (1991) The COUP-TFs compose a family of functionally related transcription factors. *Gene Expr.* **1**, 207-216.

Wasylyk, B., Hahn, S.L., and Giovan, A. (1993) The Ets family of transcription factors. *Eur. J. Biochem.* **211**, 7-18.

Watt, S.M., and Vasser, J.W.M. (1992) Recent advances in the growth and isolation of primitive human haematopoietic progenitor cells. *Cell Prolif.* **25**, 263-297.

Weening, R.S., Roos, D. and Loos, J.A. (1974) Oxygen consumption of phagocytosing cells in human leukocyte and granulocyte preparations: a comparative study. *J. Lab. Clin. Med* **83**, 570-576.

Weening, R.S., Wever, R. and Roos, D. (1975) Quantitative aspects of the production of the production of superoxide radicals by phagocytosing human granulocytes. *J. Lab. Clin. Med* **85**, 245-252.

Weening, R.S., Adriansz, L.H., Weemaes, C.M., Lutter, R., Roos, D. (1985) Clinical differences in chronic granulomatous disease patients with cytochrome b-negative or cytochrome b-positive neutrophils. *J. Pediatr.* **107**, 102-104.

Weisbrod, S. (1982) Active chromatin. *Nature* **297**, 289-295.

Weiss, S.J. (1989) Tissue destruction by neutrophils. *N. Engl. J Med.* **320**, 365-376.

Weitzman, M.D., Kyostio, S.R.M., Kotin, R.M., and Owens, R.A. (1994) Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. *Proc. Natl. Acad. Sci. USA* **91**, 5808-5812.

Whitcomb, J.M., and Hughes, S.H. (1992) Retroviral reverse transcription and integration: progress and problems. *Annu. Rev. Cell Biol.* **8**, 275-306.

White, J.M. (1993) Integrins as virus receptors. *Curr. Biol.* **3**, 596-599.

Wickham, T., Mathias, P., Cheresh, D., and Nemerow, G. (1993) Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_3$ promote adenovirus internalisation but not virus attachment. *Cell* **73**, 309-319.

Wientjes, F.B., Hsuan, J.J., Totty, N.F. and Segal, A.W. (1993) p40*phox*, a third cytosolic component of the activation complex of the NADPH-oxidase to contain SH3 domains. *Biochem. J.* **296**, 557-561.

Williams, D.A. (1993) Ex vivo expansion of hematopoietic stem and progenitor cells-robbing Peter to pay Paul? *Blood* **81**, 3169-3172.

Wood, P.M. (1987) The two redox potentials for oxygen reduction to superoxide. *TIBS* **12**, 250-251.

Woodman, R.C., Ruedi, J.M., Jesaitis, A.J., Okamura, N., Quinn, M.T., Smith, R.M., Curnutte, J.T. and Babior, B.M. (1991) Respiratory burst oxidase and three of four oxidase-related polypeptides are associated with the cytoskeleton of human neutrophils. *J. Clin. Invest.* **87**, 1345-1351.

Woodman, R.C., Erickson, R.W., Rae, J., Jaffe, H.S. and Curnutte, J.T. (1992) Prolonged recombinant interferon-gamma therapy in chronic granulomatous disease: Evidence against enhanced neutrophil oxidase activity. *Blood* **79**, 1558-1562.

Wu, D,-d., and Keating, A. (1993) Hematopoietic stem cells engraft in untreated transplant recipients. *Exp. Hematol.* **21**, 251-256.

Wymann, M.P., von Tscharner, V., Deranleau, D.A. and Baggiolini, M. (1987) Chemiluminescence detection of H_2O_2 produced by human neutrophils during the respiratory burst. *Anal. Biochem.* **165**, 371-378.

Xu, L.C., Young, H.A., Blanco, M., Kessler, S., Roberts, A.B., and Karlsson, S. (1994) Poor transduction efficiency of human hematopoietic progenitor cells by a high titre amphotropic retrovirus producer cell clone. *J. Virol.* **68**, 7634-7636.

Yacobson, B., Koch, T., and Winocour, E. (1987) Replication of adeno-associated virus in synchronised cells without the addition of helper virus. *J. Virol.* **61**, 972-981.

Yacobson, B., Hrynko, T.A., Peak, M.J., and Winocour, E. (1989) Replication of adeno-associated virus in cells irradiated with UV light at 254nm. *J. Virol.* **63**, 1023-1030.

Yang, Q., Chen, F., and Trempe, J.P. (1994) Characterisation of cell lines that inducibly express the adeno-associated virus Rep proteins. *J. Virol.* **68**, 4847-4856.

Yang, Y., Nunes, F.A., Berencsi, K., Furth, E.E., Gonczol, E., and Wilson, J.M. (1994) Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA* **91**, 4407-4411.

Yong, E.C., Klebanoff, S.J. and Kuo, C.C. (1982) Toxic effect of human polymorphonuclear leukocytes on Chlamydia trachomatis. *Infect. Immun.* **37**, 422-426.

Youngson, C., Nurse, C., Yeger, H., Cutz, E. (1993) Oxygen sensing in airway chemoreceptors. *Nature* **365**, 153-155.

Yu, S.F., Von Ruden, T., Kantoff, P.W., Garber, C., Seiberg, M., Ruther, U., Anderson, W.F., Wagner, E.F., and Gilboa, E. (1986) Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Proc. Natl. Acad. Sci. USA* **83**, 3194-3198.

Yu, V.C., Delsert, C., Anderson, B., Holloway, J.M., Devary, O.V., Naar, A.M., Sung Yun Kim., Boutin, J-M., Glass, C.K., and Rosenfeld, M.G. (1991) RXRβ: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* **67**, 1251-1266.

Zhang, D., Hetherington, C.J., Hui-Min Chen, Tenen, D.G. (1994) The macrophage transcription factor PU1 directs tissue specific expression of the macrophage colony-stimulating factor receptor. *Mol. Cell. Biol.* **14**, 373-381.

Zhen, L., King, A.A., Xiao, Y., Chanock, S.J., Orkin, S.H., and Dinauer, M.C. (1993) Gene targeting of X chromosome-linked chronic granulomatous disease locus in a human myeloid leukemia cell line and rescue by expression of recombinant gp91*phox*. *Proc. Natl. Acad. Sci. USA.* **21**, 9832-9836.

Zhou, S.Z., Broxmeyer, H.E., Cooper, S., Harrington, M.A., and Srivistava, A. (1993) Adeno-associated virus 2-mediated gene transfer in murine haematopoietic progenitor cells. *Exp. Hematol.* **21**, 928-933.

Zhou, S.Z., Cooper, S., Kang, L.Y., Ruggieri, L., Heimfeld, S.,Srivistava, A., and Broxmeyer, H.E. (1994) Adeno-associated virus 2-mediated high efficiency gene transfer into immature and mature subsets of haematopoietic progenitor cells in human umbilical cord blood. *J. Exp. Med.* **179**, 1867-1875.